DECLARATION OF **KIERAN SCOTT, PH.D.** **UNDER 37 C.F.R. §1.132** Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket No.	47-216
	Confirmation No.	7135
	First Named Inventor	GRAHAM, GARRY GEORGE
	Application Number	10/517,256
	Filing Date	June 10, 2003
	Group Art Unit	1643
	Examiner Name	Rawlings, Stephen L.
	Title: "METHOD OF INHIBITING PROSTATE CANCER CELL PROLIFERATION"	

Sir:

1. I, Kieran Scott, Ph.D declare and say I am a resident of Eastgardens, New South Wales, Australia.

2. I am an employee of the University of New South Wales and am a Senior Research Fellow. Details of my career as well as publications may be found in my curriculum vitae (Exhibit 1).

Considered
SR 9/13/2010

3. I have been asked by FB Rice & Co, my Patent Attorneys, to provide an opinion on the state of knowledge surrounding the inhibition of sPLA2-IIA enzyme activity in the treatment of prostate cancer in 2002 and on the invention described in US 10/517,256 (referred to below as the "Patent Application"). I have been asked in particular to comment on the obviousness rejections set out in the Office Action dated 12 February 2010 issued in connection with the Patent Application.

4. I am an inventor of the invention described in the Patent Application.

5. I have read the Patent Application and have reviewed the claims that I understand are presently being considered by the United States Patent and Trademark Office. I understand that the claimed invention (referred to hereafter as the "Invention") relates to a method of inhibiting prostate cancer cell proliferation. I understand that claim 1 as proposed to be amended is limited to treatment of prostate cancer patients who have been subject to androgen ablation therapy. The importance of this invention is that it provides,

for the first time, a method of inhibiting the sPLA2-IIA-mediated proliferation of prostate cancer cells in late stage prostate cancer.

6. I have reviewed the Office Action dated 12 February 2010 in relation to the above-referenced Patent Application. I understand that the Patent Office has taken the position that the Invention would be obvious to a person skilled in the art in light of Graff *et al.* (2001), when combined with the disclosures of Church *et al.* (2001), Attiga *et al.* (2000), Liu *et al.* (2000), or Kelavkar *et al.* (2001).

7. Graff *et al.* is cited as disclosing that "enhanced sPLA2-IIA expression may be involved in the malignant progression of human prostate cancer and suggests that specific inhibitors of the group IIA sPLA2 may be useful for prostate cancer chemotherapy." Church *et al.* is cited as disclosing cyclic peptide inhibitors of sPLA2-IIA. Attiga *et al.* is cited as disclosing that invasion of prostate cancer cells (i.e. PC-3 and DU-145 cells) is inhibited by a PLA2 inhibitor, a general COX inhibitor and a selected COX-2 inhibitor. Liu *et al.* is cited as disclosing a COX-2 inhibitor. Kelavkar *et al.* is cited as disclosing a 15-lipoxygenase inhibitor.

8. It is my understanding that the Patent Application and the cited publications are to be viewed from the perspective of one of ordinary skill in the art in the relevant field (a "Skilled Person") at the time of filing of the Patent Application in question. I have been asked to consider this time to be the period around or before 7 June 2002 (the "Relevant Period"). I would expect a Skilled Person in the field of arachidonic acid metabolism in prostate cancer during the Relevant Period to have been represented by a scientist with a Ph.D. degree in Biochemistry and/or at least 3 to 5 years experience in the field of Biochemistry, or an educational background at the same degree level in a related field and equivalent level of experience.

9. I am very familiar with the technical field of the claimed invention. I am qualified to analyze literature in this field and to provide my opinion as to what literature in this field discloses or suggests to the Skilled Person at the Relevant Period.

10. By the Relevant Period I had attained at least the level of such a Skilled Person, and further in view of my qualifications discussed above, I believe that I am qualified by training and experience to address what a Skilled Person would have understood from reading the Patent Application and the cited publications.

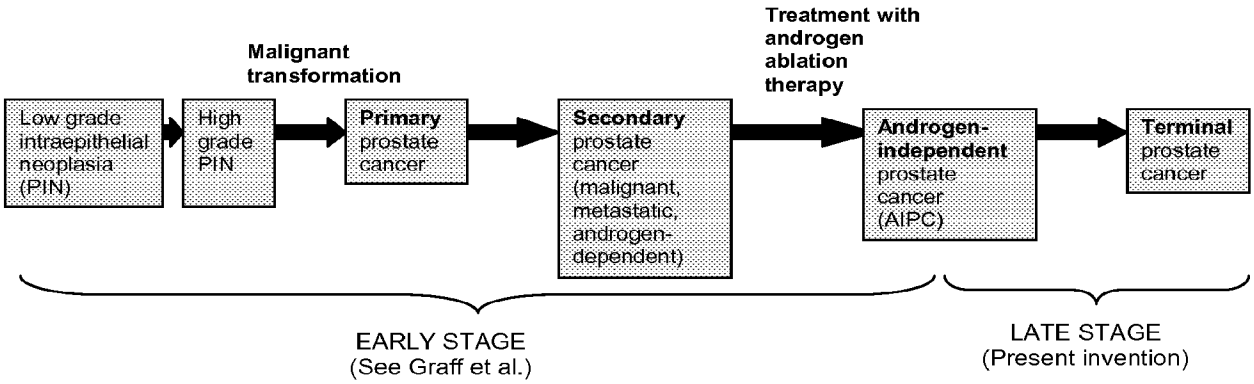
11. None of the documents referred to in paragraphs 6 and 7 above suggest to me now or would have convinced me in June 2002 that inhibiting sPLA2-IIA-mediated proliferation of prostate cancer cells would produce a successful treatment for late stage prostate cancer. These documents are at best a collection of papers describing inhibitors of unrelated enzymes, with a paper describing that sPLA2-IIA expression is elevated in malignant progression and progression to androgen-independence and a paper describing cyclic peptide inhibitors of sPLA2-IIA thrown into the mix.

12. Further, if it had been suggested to me before the present invention that inhibiting the sPLA2-IIA-mediated proliferation of prostate cancer cells after androgen ablation therapy would produce a successful treatment for late stage prostate cancer I would have been skeptical based on my knowledge at the time of the role of sPLA2-IIA in prostate cancer.

Overview of progression of prostate cancer

13. Figure 1 (shown below) is a schematic diagram of the main events in the overall progression of prostate cancer. It is well known in the art that malignant progression of prostate cancer cells (i.e. early stage prostate cancer) is both biochemically and functionally distinct from late stage (terminal) prostate cancer.

Figure 1.



14. Malignant progression is defined as the process by which cells progress from being benign (i.e. not malignant or harmful) to a state of being malignant. Malignancy is defined in oncology as a state in which cancer cells invade and destroy nearby tissue and spread (metastasize) to other sites in the body. It is recognised that cancer cells must acquire at least six functionally separable capabilities to be tumorigenic. Tissue invasion

and metastasis is just one of several necessary additional functional capabilities that must be acquired by tumours for them to be malignant. This function requires the activation of classes of molecules such a secreted proteases (eg matrix metalloproteases) and cell-cell adhesion molecules (e.g. E-cadherin) that are not required for the well characterised cell cycle machinery that regulates cell proliferation (Hanahan and Weinberg (2000); Exhibit 2).

15. Local tissue invasion is followed by metastasis, thus completing the process of malignant progression. When tumor cells metastasize, the new tumor is called a secondary or metastatic tumor. At this stage, on diagnosis, most prostate cancers are still androgen dependent and are treated by either chemical or surgical androgen ablation therapy. Progression to androgen independence subsequently occurs and patients with androgen-independent prostate cancer are described as having hormone refractory prostate cancer (HRPC) at this stage because they fail to respond to antiandrogen therapy. Progression to androgen independence in clinical cases therefore occurs after malignant progression (i.e. after tissue invasion and metastasis) has begun.

The present invention compared to Graff *et al.*

16. The Patent Application is directed towards (i) the inhibition of proliferation of prostate cancer cells and (ii) in cells of subjects who have been subjected to androgen ablation therapy (see Example 1 of the Patent Application). In other words, the Patent Application relates to the inhibition of the proliferation of prostate cancer cells following androgen ablation therapy after progression to androgen-independence has occurred (i.e. treatment of late stage prostate cancer).

17. There is nothing in Graff *et al.* that suggests that inhibition of sPLA2-IIA enzyme activity would produce a successful treatment for late stage prostate cancer as claimed in the Patent Application, i.e. following androgen ablation therapy and after progression to androgen-independence has occurred (see Figure 1 above).

18. Graff *et al.* is clearly directed towards malignant progression of prostate cancer, i.e. early stage prostate cancer, as outlined in Figure 1 above (see for example Conclusions, page 3857; Results and Discussion page 3859; and last paragraph of Results and Discussion on page 3860 of Graff *et al.*).

19. At most, Graff *et al.* suggests that inhibitors of sPLA2-IIA may be useful in treatment of early stage prostate cancer – in other words, to inhibit progression of malignancy and progression to androgen-independence. There is nothing to suggest that these inhibitors would be useful after progression to malignancy and androgen independence has occurred. At that stage, the Patent Application shows it becomes necessary to target a different biochemical mechanism such as cell proliferation. There is no evidence in Graff *et al.* to suggest that inhibitors of sPLA2-IIA would be effective at inhibiting proliferation of prostate cancer cells that have already progressed to malignancy and androgen independence.

20. There is nothing in Graff *et al.* to suggest that inhibition of the proliferation of prostate cancer cells will be a successful treatment for late stage prostate cancer, as claimed in the Patent Application.

21. Malignant progression and progression to androgen-independence (as discussed in Graff *et al.*) are both biochemically and functionally separable from the process of cellular proliferation (as claimed in the Patent Application). For example, aberrant cell proliferation, though necessary, is not sufficient for malignant progression.

22. Tumour growth is the result of a balance between the rate of cell proliferation and the rate of programmed cell death (apoptosis). Tumour growth can therefore be increased not only by increasing the rate of cell proliferation (as the present inventors have shown for sPLA2-IIA), but by changes that reduce the rate of apoptosis.

23. On this point, the reference 29, cited in the last paragraph of Graff *et al.* (Denmeade *et al.*, 1996; Exhibit 3) shows that a major determinant of increased tumour growth in prostate cancer may not be increased rate of proliferation but enhanced cell survival due to suppression of cell death mechanisms (apoptosis). This prior art, cited in the last paragraph of Graff *et al.* therefore would suggest to the Skilled Person to try a known inhibitor of apoptosis as a treatment for prostate cancer.

24. The last paragraph of Graff *et al.* states: "This report therefore provides compelling evidence that enhanced sPLA2-IIA expression may be involved in malignant progression of human prostate cancer and suggests that specific inhibitors of the group IIA sPLA2 may be useful for prostate cancer chemotherapy."

25. This conclusion is based on findings that: (i) "the expression of Group IIA sPLA2 is specifically increased with progression of human prostate cancer cells to androgen independence"; (ii) "sPLA2-IIA expression is dramatically increased in primary, high-grade prostate cancers"; (iii) "this increase is related to the increased proliferative index that typifies the more advanced CaPs (26, 29)"; and (iv) "sPLA2-IIA expression is inversely related to 5-year patient survival".

26. Finding (i) by Graff is based on observations that the sPLA2-IIA expression levels are increased in androgen independent variants relative to their androgen-dependent parent cells. The androgen independent variants have been derived by selection either for androgen independent growth in culture or have been selected for androgen-independent growth of androgen-dependent tumours in immune-deficient mice (following castration). While the cell lines examined in Graff satisfy the criteria for progression to androgen independence in culture, it is important to note, they have not been directly derived from patients who have been subjected to hormone ablation therapy, but have been derived from androgen-dependent prostate cells by *ex vivo* manipulation. They therefore do not directly derive from hormone refractory patients and thus do not address the issue of whether sPLA2-IIA remains upregulated in patients who have been subjected to hormone ablation therapy.

27. The data in the Patent Application identify sPLA2-IIA as remaining upregulated in patients following hormone ablation therapy and thus link treatment with inhibitors directly to patients post hormone ablation therapy. Because, in these studies, the cancer cells have also failed to be removed by hormone ablation therapy they have very likely already undergone progression to androgen independence. In addition, the biochemical mechanisms by which progression to androgen independence occurs are highly heterogeneous, with at least five different pathways by which androgen independence can develop being recognised (Feldman and Feldman (2001); Exhibit 4). Thus *in vitro* progression to androgen independence of clonal cells in culture (as discussed in Graff *et al.*) is not necessarily predictive of progression to androgen independence *in vivo*. The Patent Application shows that sPLA2-IIA levels remain elevated in prostate tissues post-hormone ablation therapy (androgen withdrawal), thus establishing that sPLA2-IIA remains elevated in the clinical entity hormone refractory prostate cancer, rather than the laboratory entity of androgen independence.

28. In contrast to the cell culture studies in Graff *et al.*, the studies on tissue levels of sPLA2-IIA in Graff *et al.* relate to malignant progression if you compare patients with benign prostate hyperplasia (BPH) (non-malignant cells) relative to patients with low grade or high grade localised cancer (malignant cells). However, these tissue studies do not relate to progression to androgen independence since the androgen status of the patients is unknown. Rather, the studies on tissue levels of sPLA2-IIA described by Graff *et al.* relate to malignant progression in comparing patients with BPH relative to patients with primary cancer.

29. Finding (ii) by Graff is based on comparison of benign prostatic hyperplasia tissues (non-malignant) with "low" grade (mean Gleason score 4.6) and "high" grade (mean Gleason score 7.0) primary tumours. Again, these studies relate to early stage prostate cancer.

30. Finding (iii) is based on the observation that a marker of cell proliferation (Ki67) is higher in prostate tissues that showed uniform sPLA2-IIA compared to prostate tissues that showed focal staining. It should be noted that of the 19 tissues with uniform staining, 8/19 (42%) were from patients with benign prostate hypersplasia (BPH) (non-malignant) (Table I). Thus the association with proliferation index does not in fact relate to malignant progression at all, since almost half of the tissues in the group studied are non-malignant. The relationship between "uniform sPLA2-IIA staining" and proliferation index could equally apply to non-malignant tissues. It is clear that this analysis in Graff *et al.* does not represent advanced prostate cancers, but a combination of benign prostatic hyperplasia (BPH) and cancer tissues.

31. Further, reference 29, cited in the last paragraph of Graff *et al.* to back up the conclusion in (iii), teaches that "The transition of late-stage high-grade PIN cells into growing localised prostatic cancer cells involves no further increase in Kp [i.e. the rate of proliferation] but is due to a decrease in Kd" [i.e. the rate of cell death/apoptosis] (page 258, column 1, para 2., Denmeade *et al.*, 1996; Exhibit 3). Accordingly, the references quoted in the final paragraph of Graff *et al.* in fact would suggest to the Skilled Person that targeting cell proliferation after malignancy progression would not be useful in treating late stage prostate cancer.

32. Finding (iv) does not show or suggest that inhibition of sPLA2-IIA-mediated proliferation will be of benefit, since as outlined above there are several ways tumours can be induced to regress that do not involve suppression of proliferation.

33. In fact, subsequent publications by Graff's research group have shown that elevation of sPLA2-IIA expression occurs even earlier than the primary cancer stage, before malignant progression (see Jiang *et al.* 2002; Exhibit 5). Exhibit 5 shows that sPLA2-IIA expression is elevated in low grade and high grade prostatic intraepithelial neoplasia (PIN), a condition which occurs in the early stages of prostate cancer (see Figure 1 above). This simply confirms what I believe a Skilled Person would understand from Graff *et al.* that sPLA2-IIA expression is elevated during the early stage of prostate cancer and is involved in progression toward malignancy and androgen independence. There is nothing in Graff et al. to suggest that sPLA2-IIA is elevated or would be a useful target in late stage cancer when progression to malignancy and androgen independence is already complete and other biochemical processes come into play.

34. I believe the Skilled Person in the art would not have been motivated to treat late stage, prostate cancer in patients who have been subjected to androgen ablation therapy based on the findings of Graff *et al.* The Patent Application is directed to treating a completely different class of patients (i.e. terminal/late prostate cancer patients who have been subjected to androgen ablation therapy) than the class of patients to which Graff *et al.* relates (i.e. prostate cancer patients with early stage prostate cancer who have not been subjected to androgen ablation therapy). As such, Graff *et al.* would not have motivated the Skilled Person to use inhibitors of sPLA2-IIA to treat late stage prostate cancer in prostate cancer patients who have been subjected to androgen ablation therapy.

35. Attiga *et al.* shows that invasion of prostate cancer cells is inhibited by a PLA2 inhibitor, a general COX inhibitor and a selected COX-2 inhibitor. Liu *et al.* relates to a COX-2 inhibitor and Kelavkar *et al.* relates to a 15-lipoxygenase inhibitor. A Skilled Person would not have found it obvious to take the findings of any of these references, which relate to completely different enzymes, alone or in combination with Graff *et al.* or Church *et al.*, with a reasonable expectation of successfully arriving at a method for treating sPLA2-IIA-mediated proliferation of late stage prostate cancer in patients who have been subjected to androgen ablation therapy.

36. Finally, the Patent Application is directed towards inhibition of the ability of the sPLA2-IIA polypeptide to catalyse the hydrolysis of membrane phospholipids at the sn-2 position to release fatty acids and lysophospholipids. Neither Graff *et al.*, nor any of the Attiga, Liu, Kelavkar or Church references, show or suggest which activity of the enzyme must be inhibited, as presently claimed. Further, neither Graff *et al.*, nor any of the Attiga, Liu, Kelavkar or Church references, show or suggest which form of the enzyme should be inhibited, as presently claimed.

37. In view of all of the above, I believe that the claimed subject matter of the Patent Application would not have been obvious to a Skilled Person at the Relevant Period.

38. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the Patent Application or any patent issuing thereon.

22nd June 2010
Date Name: Kieran F. Scott PhD

Curriculum Vitae: Dr Kieran F. Scott (April, 2010).

1. Name and contact details.

SCOTT, Dr Kieran Francis

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2. Citizenship, Date of Birth, Gender

Citizenship: Australian and New Zealand
DOB: 1st April, 1957.
Gender: male

3. Academic qualifications

PhD in Genetics. Australian National University. Awarded 1983.
BSc (Hons I) Massey University New Zealand. Awarded 1979.

4. Current Appointment

2007- Present. Senior Research Fellow (0.3 FTE), St Vincent's Hospital Clinical School, The University of New South Wales.

5. Previous appointments

1983. Visiting Fellow, Centre for Recombinant DNA Research, Research School of Biological Sciences. Australian National University.

1984. Postdoctoral Fellow, Genetics Department, Research School of Biological Sciences, Australian National University.

1985-1988. Research Fellow, Genetics Department, Research School of Biological Sciences, Australian National University.

1988-1991. Senior Scientist, Pacific Biotechnology Ltd., Sydney.

1992. Research Fellow, Garvan Institute of Medical Research, Sydney.

1992-2000. Senior Research Fellow, St Vincent's Clinical School, UNSW

2000-2001. Senior Research Officer Arthritis Programme, Garvan Institute of Medical Research.
2002-2006. Senior Research Fellow, St Vincent's Clinical School, UNSW
2006-2007. Senior Research Fellow (Honorary), St Vincent's Clinical School, UNSW

6. NHMRC research support (Total Support, $ 1.653M)

1993-1995 The production of a recombinant immunotoxin with potential utility in leukaemia therapy. $43,000 p.a. Chief Investigators, A/Prof. K Atkinson, Prof. J. Biggs, Dr. P. Kearney, **Dr. K. F. Scott**.

1993-1995. The study of phospholipase A_2 in labour onset. $100,000 p.a. Chief Investigators: Dr. G. Rice, **Dr K. Scott,** Prof. S. Brennecke and Prof. G. Thorburn.

1995-1997. The role of human type II secretory phospholipase A_2 in rheumatoid arthritis. $103,000 p.a. Chief Investigators, **Dr K.F. Scott** and Prof. P.M. Brooks.

1998-2000 Modulation of cytokine-mediated inflammatory responses by human type II phospholipase A_2. $130,000 p.a. Chief Investigators: **Dr K F Scott** and Prof P Brooks

2003-2005. Mechanism of action of secreted phospholipase A_2 and its inhibition in inflammation. $175,000p.a. Chief Investigators, **Dr K.F. Scott,** Prof. G.G. Graham, A/Prof H.P. McNeil.

7. Research support from other sources. (Total Support $6.42M)

1993. Arthritis Foundation of Australia Grant in Aid. Phospholipase A_2 in arthritis. $6,000. **Dr. K.F. Scott**, Prof. P.M. Brooks.

1993. ARC Small Grant Scheme. Structure-function studies on secretory phospholipase A_2. $12,000. **Dr. K.F. Scott** and Dr. P. Curmi.

1993. Rebecca Cooper Foundation. Phospholipase A_2 in arthritis. $ 10,430. **Dr. K. F. Scott** and Prof. P.M. Brooks.

1994. ARC Small Grant:- Structure-function studies on secretory phospholipase A_2. $12,000. Chief Investigators, **Dr. K. F. Scott** and Dr. P. Curmi.

1995-1997 Syndicated R&D Programme:- Phospholipase A_2 inhibitors. Chief Investigators, **Dr K. F. Scott**, Prof. P. Brooks and Prof. J. Shine $1.03M p.a.

1996 UNSW Capital Grant:- Macromolecular crystallographic analysis facility. $815,000. Dr P. Curmi, **Dr K. Scott** *et al.*

1996 Small ARC grant:- Transcriptional control of secretory phospholipase A_2 gene expression. $10,000. **Dr K. Scott**

1996 Viertel Foundation. Molecular modelling equipment. $100,000. Prof. R.M. Graham, Dr P. Reik, Dr. D. Ogg, Dr B. Church, **Dr K.F. Scott.**, Prof. P. Brooks, Dr T. Iismaa, Dr H. Herzog, Prof. J. Shine.

1997 UNSW Capital Grant:- Resources for structural biology. $250,000. Dr. P. Curmi and **Dr K.F. Scott**.

1997 Rebecca Cooper Foundation:- $11,000. Transcriptional control of secretory phospholipase A_2 gene expression. **Dr K. F. Scott**.

2000 Glaxo-Wellcome: $35,000. Detection of transcripts encoding enzymes of the eicosanoid biosynthetic pathway in cells from human blister fluids. **Dr K.F. Scott**

2000-2001. Smithkline Beecham (Australia and UK) $70,000. The effect of paracetamol on prostaglandin production by human rheumatoid synovial cells in culture. Dr G.G. Graham and **Dr K. F. Scott**

2001-2002 GlaxoSmithkline (Australia and UK) Dr G.G. Graham and **Dr K. F. Scott** $50,000 The effect of paracetamol on prostaglandin production by human rheumatoid synovial cells in culture.

2001-2002. Rebecca Cooper Foundation. $15,000. **Dr K. F. Scott.** Research on phospholipase A_2.

2004-2006. Department of Veterans Affairs. Dong Q, **Scott K. F.**, Graham G and Russell PJ: $490,500. Oncogenic action and therapeutic potential of PLA_2 in prostate cancer.

2007-2009. NSW Cancer Council. **Scott K. F.**, Graham, G. G., Dong, Q., Russell, P. J., $300,000 Secreted phospholipase A_2 in prostate cancer.

2009. St George Medical Research Foundation. Galettis, P. **Scott, K. F.** Graham, G.G., Liauw, W., De Souza, P. $20,000 A Quantitative assay for novel compounds that show promise as oral treatments for advanced prostate cancer.

2009. UNSW Major Research Equipment and Infrastructure Initiative. Power, C, Housley, G., Apte, M., Bertrand, P., Carrive, P., Crowe, P., Gunning, P., Haber, M., Hardeman, E., Hogg, P., Grimm, M., Geczy, C., Kavalaris, M., Khachigian, L., Lock, R., McNeil, P., **Scott, K. F.,** Walsh, W., Yang, L., Rae, C. $769,000 Positron Emission Tomographic (PET) scanner and associated infrastructure.

2010-2012. Prostate Cancer Foundation of Australia. de Souza, P. **Scott, K. F.** Young, P. , Graham, G. G., Liauw, W., Russell, P. J. $450,000. A translational and pharmacokinetic study of a novel orally-active, targeted treatment for hormone refractory prostate cancer.

8. Competitive Industry grants (Total Support $2.1M)

1988. Generic Technology Grant from the Department of Industry Technology and Commerce (DITAC) in 1988 entitled "Recombinant biosynthesis of multi-subunit proteins requiring post-translational modification for biological activity". $1,004,025. Principal Investigator, Dr. K.F Scott. Associate Investigators, Dr. L. Lazarus, Dr. J. Shine, Dr. P Gray, Dr. M. Stuart.

1990. Discretionary Grant from DITAC entitled "Therapeutic monoclonal antibody for treatment of septic shock". $1,080,050. Project Leader, Dr. K.F.Scott, Principal Researchers Dr. A. Protter, Prof. P. Gray, Dr. I. Rajkovic.

9. Publications (Total citations, 1391, average citation rate 26.75, H-index 23)

42 original papers in peer-reviewed journals
12 invited reviews
2 book chapters
19 conference proceedings papers
89 presentations at meetings.
17 patents:- 11 patents issued, 1 pending, 5 filed/lapsed

10. Teaching Experience.

My academic career in research has been intimately associated with the teaching life of the Universities I have been associated with, particularly in the training of graduate and post-graduate students. During my early years as a postgraduate student and post-doc at the Centre for Recombinant DNA Research at the ANU, I developed and lectured in training courses in both theoretical and practical aspects of molecular biological techniques. I was a guest lecturer in the Department of Botany, ANU and gave several lectures per year in a variety of courses. During my post-doctoral years at ANU I directly supervised three PhD students (see Detailed CV), all of whom remain in research and/or teaching at Australian Institutions. I also supervised three honours students in this time. During my time in industry I cosupervised two PhD students and since returning to academia in 1992 I have supervised four PhD students, one of whom won the Campion Ma Playoust Award for his thesis presentation from the Australian Society for Medical Research. I have supervised four Masters students and four Honours students. Since 2002, I have assisted in the design and execution of practical courses for the Masters in Biopharmaceutical development, UNSW and have been a mentor and examiner for students in the UNSW postgraduate course "Molecular Basis of Disease".

11. Research Experience

My early research training was in molecular genetics in the laboratory of Prof. John Shine. I developed a novel method for cloning and characterising bacterial genes involved in plant-microbe interactions using random insertional mutagenesis with the transposable element Tn5 (Journal Article 4). The physical properties of Tn5 enabled the DNA flanking the site of insertion of the transposon to be cloned and sequenced. The wild-type allele could then be isolated from a genomic library of bacterial DNA and used to correct the phenotype of the mutant bacterium. These experiments were one of the earliest examples of the demonstration of Koch's postulates using molecular techniques. This system continues to be widely used in bacteria and approaches like this

were the conceptual forerunners to the development of gene knockout techniques in animals. I was the first to clone and characterise the genes encoding nitrogenase, the central enzyme in the entry of elemental nitrogen into the biosphere. (Journal Articles 1, 6, 7, 9) Through these studies I gained experience in gene cloning and characterisation, bacterial genetics, and plant biochemistry. I used this experience in my post-doctoral period to clone and characterise genes involved in the recognition of non-legume plants by bacteria. This work resulted in the discovery of a new nodulation gene nodK (Journal Article 10). These studies also resulted in the issuing of several US patents, in particular, one that described the development of a bacterial strain that resulted in increased plant yield over its parent strain, a key goal of genetic manipulation studies in biological nitrogen fixation.

In 1988 I changed careers and moved to industry. My research interests were in the area of understanding the role of phospholipase A_2 (PLA_2) in the pathogenesis of inflammatory diseases such as septic shock and rheumatoid arthritis. The work was focused on the development of useful antibody-based PLA_2 antagonists and has led to some exciting basic discoveries. We expressed a newly identified human group IIA secreted PLA_2 (hGIIA), developed a purification strategy for hGIIA and raised murine monoclonal antibodies to both linear and conformational epitopes of the molecule (Conference Proceedings 19). Two of these antibodies formed the basis for the development of a sensitive and specific sandwich ELISA which has been used to quantify hGIIA levels in circulation of patients with arthritis (Journal articles 18,19) and septic shock (Journal articles 17, 20). This study also showed for the first time that the PLA_2 activity associated with the onset and severity of septic shock is identical to that associated with the severity of rheumatoid arthritis. The ELISA we developed is in use in several laboratories around the world. Recently, with the discovery of at least eleven human forms of secreted PLA_2 and the observation that our ELISA is specific for the hGIIA form (Review 2), the clinical studies described here remain important in the field, identifying this form as being associated with inflammatory conditions in humans. Two of our antibodies neutralise $sPLA_2$ in *in vitro* activity assays (Conference proceedings 18,19). The antibodies also show positive effects on blood pressure in a baboon model of severe septic shock (unpublished data). This work led to the view that hGIIA is a contributor to the pathophysiology of septic shock and may be implicated in the progression and severity of other immune-mediated inflammatory disorders with which it has been clinically associated including asthma, Crohn's disease, psoriasis and more recently, coronary artery disease and prostate cancer. During this time in industry I gained experience in mammalian and bacterial expression systems, scale-up production, purification and analysis of recombinant proteins, production and screening of monoclonal and polyclonal antibodies, antibody-based assays, animal models of inflammation in rabbits, rats, mice and baboons and experience in lipid mediator and enzyme biochemistry.

I moved back to academic research in 1992 to continue my research interest in understanding the mechanism of PLA_2 action in the pathogenesis of rheumatoid arthritis. In collaboration with Prof. Peter Brooks I have gained experience in the design, execution and analysis of clinical studies with arthritis patients (Abstract 37). Using immunohistochemistry, confocal microscopy (through collaboration with Prof. Anne Cunningham) and *in situ* hybridisation with human synovial tissue, we have shown that the induction of hGIIA protein expression in the synovium is associated with histological markers of inflammation (journal article 34). Through collaboration with Prof, Pruzanski and Dr Vadas at the Wellesley Hospital, Toronto and Dr Greg Rice, Royal Women's Hospital, Melbourne I quantified the levels of hGIIA in various diseases and in association with labour onset. These studies have established the Group IIA isoform of PLA_2 as a predominant secreted form in these conditions. I have also gained experience in collagen-induced arthritis models in rats and mice and carrageenan-induced acute inflammation models in rats. These models

were used in the preclinical evaluation of peptide-based agents that I performed under contract to industry (unpublished data and Abstract 50).

I have further developed my protein biochemistry experience and knowledge in the area of lipid mediators. A highlight of this work was the discovery of synthetic peptide inhibitors of hGIIA (Journal Article 28). These studies identified that synthetic peptides derived from the native sequence of hGIIA from different species were specific inhibitors of the enzyme from which they were derived, a process we have termed "native peptide inhibition". Recent developments in this work have generated potent cyclic peptide inhibitors of hGIIA function (Abstracts 63, 65, 67, 69, 71, Journal Article 38). These studies are particularly important since they raise the possibility of developing isoform-specific $sPLA_2$ inhibitors that may be useful in defining the relative roles of the eleven known human $sPLA_2$ variants. This work, in collaboration with Dr Bret Church (now with the Department of Pharmacy, University of Sydney), Dr Albert Tseng and Adam Inglis, has given me experience with molecular modelling, surface plasmon resonance and further developed my experience in protein and peptide biochemistry.

My work on the mechanism of action of hGIIA in rheumatoid fibroblast cell function, has shown that exogenous hGIIA, at concentrations found in the synovial fluid of patients with arthritis, upregulates TNF-α-stimulated PGE_2 production by superinduction of the $cPLA_2$-α/cyclooxygenase-2 pathway (Abstracts 61, 64, Bidgood *et al.*, J. Immunol, 2000). The mechanism of this superinduction is completely unknown. Further, hGIIA alone upregulates COX-2 without enhanced PGE_2 release or induction of the known pathways involved in COX-2 upregulation such as the p38 mitogen activated kinase pathway of nuclear factor-κB mobilisation (Abstract 70, Bryant *et al.*, manuscript submitted). Rather, hGIIA activates the ERK mitogen activated kinase pathway in these cells. Given the known role of the ERK pathway in activation of gene expression, these findings suggest that hGIIA may have broader effects on synovial cell function than upregulation of the prostaglandin pathway alone (Abstract 72). Further, we have recently shown that hGIIA is rapidly internalised by rheumatoid synovial fibroblasts, raising the possibility that $sPLA_2$ effects may be mediated by a receptor-dependent mechanism. It is important to define this pathway in synovial fibroblasts since it may be a novel mechanism by which hGIIA and possibly other secreted proteins, may modulate intracellular signal transduction pathways. These studies have given me experience and further developed my knowledge of cytokine signalling pathways, cytoskeletal cell biology, endocytotic pathways, confocal microscopy, primary cell culture, gel shift assays and the use of phospho-specific antibody techniques.

In the last several years I have established a successful collaboration with Prof Garry Graham, Department of Physiology and Pharmacology, UNSW which has attracted industry support for our studies on the mechanism of action of the commonly-used and cost-effective analgesic, paracetamol. These studies have shown that paracetamol is a potent inhibitor of cyclooxygenase-2-dependent prostaglandin synthesis in human rheumatoid synovial cells (Review 3). I have also been successful in obtaining industry support to develop methods for detecting gene expression in cells derived from human blister fluid in response to UV-induced inflammation using real time RT-PCR. I also cosupervised a PhD student (Megan Taberner) working on global gene expression in synovial cells using DNA microarrays (publication 40).

In collaboration with Dr Bret Church, my most recent PhD student, Dr Lawrence Lee has solved six X-ray crystal structures of hGIIA protein cocrystallised with both peptide and non-peptide hGIIA inhibitors. This work has identified important structural features induced by binding of inhibitors to hGIIA that may modulate newly-identified enzyme activity-independent effects of hGIIA

selectively over enzyme activity-dependent effects. These studies may lead to the design of a new class of selective hGIIA inhibitor.

In recent years, in collaboration with Dr Qihan Dong, Department of Mediciane, University of Sydney, Prof Garry Graham, and Prof Pam Russell, (Oncology Research Centre, Prince of Wales Hospital), we have identified and validated hGIIA as a target for therapy in advanced prostate cancer (publication 39). Following on from this work, we have now shown that our cyclic pentapeptide inhibitors of hGIIA can induce complete regression of androgen insensitive tumours on oral administration in a xenograft model of prostate cancer (manuscript in preparation). These findings confirm the potential of this approach as a novel treatment for patients with advanced prostate cancer, a disease that claims 3000 lives per year in Australia and we hope to initiate clinical trials of our approach in the near future. To this end we have established a collaboration with dr Paul Young, Department of Pharmacy, University of Sydney to examine formulation approaches to these compounds.

12. Detailed Curriculum Vitae

(a) Academic Background

1978 Undergraduate training in chemistry and biochemistry, Massey University, New Zealand.
1978 New Zealand Institute of Chemistry prize for Biochemistry, New Zealand.
1979 BSc (Hons I) in Biochemistry Massey University, New Zealand.
1979 Massey Scholarship, New Zealand
1979 University Grants Committee Postgraduate Scholarship, New Zealand
1980 ANU Postgraduate Scholarship, Canberra, Australia
1983 PhD in Genetics, Australian National University. PhD thesis title: Symbiotic Nitrogen Fixation in *Rhizobium*: :A Molecular and Genetic Analysis.
1984 J. G. Crawford Prize for PhD thesis, Canberra, Australia.

(b) Postgraduate training

Following my graduate training in gene cloning, I chose to continue research into the cloning of genes important in biological nitrogen fixation. This choice was made because at that time, the Research School of Biological Sciences was at the forefront of gene cloning technology and analysis in my field and I was keen to pursue the question of which bacterial genes were important for nitrogen fixation in nonlegume plants. After one year as a visiting fellow and one year as a post-doctoral fellow at ANU, I was appointed a Research Fellow in the Genetics Department of the Research School of Biological Sciences. During the three years of this appointment I further developed my gene cloning and characterisation expertise, established a number of successful collaborations, cosupervised several PhD students and was successful in obtaining independent peer-reviewed funding, notably a team leader's grant in the ARC National Research Fellowship scheme. My publication record in this period was steady with a well-cited sole author paper (Journal Article 10) published during this time. These studies also resulted in the issuing of several US patents. In particular, I am sole inventor on a patent describing a method for increasing the growth and yield of plants by inoculation with a genetically-modified bacterium which was never published in the academic literature.

In 1988 I changed fields and careers and was recruited to industry with a small startup biotechnology firm Pacific Biotechnology, Sydney. I gained experience in protein expression in bacteria and mammalian cells, protein purification and analysis, monoclonal antibody production and characterisation, ELISA and enzyme activity assay development, animal models of inflammation, particularly in rabbits, rats, mice and baboons and clinical studies in humans. These skills were used in several project areas including expression of follicle stimulating hormone, development of novel antibody-based anti-leukaemic drugs and development and preclinical trial of monoclonal antibodies to secretory phospholipase A_2. This change of field and movement to industry resulted in a break in my academic publication record for two years. Much of the work carried out in this period has not been published in the academic press.

I then moved to take up an academic research position in the Department of Medicine, St Vincent's Clinical School, The University of New South Wales. During this time I built and staffed laboratories in the Medical Professorial Unit at St Vincent's Hospital, funded primarily by a successful R&D syndicate proposal based on the development of novel inhibitors to secretory phospholipase A2 (hGIIA). These laboratories were moved to the Garvan Institute in 1997 and are now located on Level 10 of the Garvan Institute Bldg, Sydney where I becam a founding member of

the Arthritis and Inflammation Research Programme of the Garvan Institute. During this time I have extended my research into the mechanism of action of hGIIA in human rheumatoid cells (see Section 11) as well as successfully continue my association with industry through contract research described above.

(d) Collaborations

I have had successful international collaborations with Dr Jeff Seilhamer (Cofounder, Incyte Pharmaceuticals, San Francisco, CA) (Journal Article 16) who cloned $sPLA_2$ and Drs Peter Vadas and Waldemar Pruzanski, the discoverers of $sPLA_2$, at the Wellesley Hospital, Toronto (20, 21, 23, 26, 27) studying the association of $sPLA_2$ with malaria, salicylate intoxication and extending our own studies on sepsis and rheumatoid arthritis. More recently I set up a collaboration with Prof Michael Gelb, University of Washington, Seattle to pursue studies on the role of sPLA2 in prostate cancer. This collaboration ahs been particularly productive giving us access to pharmacological resources and reagents that have proved essential for progress to be made. I have had a long-standing association with Prof. Timo Nevalainen that resulted in a coauthored invited review last year.

My collaboration with Prof. S. Brennecke and Dr. G. Rice, Royal Women's Hospital, Melbourne, has been productive in the area of $sPLA_2$ in parturition (19, 24, 25, 29, 31, 32). I have also collaborated extensively with local investigators including Dr Ken Ho, Garvan Institute and Dr Richard Lee, St Vincent's Intensive Care Unit (17), Dr Robyn Ward, Oncology, St Vincent's Hospital and Dr Lou McGuigan, Rheumatology, St George Hospital (18), Prof. Reg Lord and Dr Hakan Parsson, Surgical Professorial Unit, St Vincent's Hospital (30, 35) who have provided clinical expertise and advice, Dr Roland Stocker, Heart Research Institute (33) and Dr Vince Munro, Anatomical Pathology, St Vincent's Hospital who provided advice on histological features of tissues (34). I have also collaborated with Prof. James Biggs and Dr Kerry Atkinson providing molecular biology expertise on the development of immunotoxins to treat leukaemia which included cosupervision of a PhD student, Dr Robert Seymour (Abstract 26) and cosupervised an honours student, Ms Louise Graham, with Prof. Paul Seale, Pharmacology Department University of Sydney working on $sPLA_2$ in asthma (Abstract 34).

I have had collaborations with several local investigators including Prof. Anne Cunningham, Prince of Wales Children's Hospital, (34), Prof. Garry Graham, Physiology and Pharmacology, UNSW (Cosupervisor, Ms Sally-Ann Robins, Abstract 73, Review 3), A/Prof. David Walsh (Abstract 74, manuscript in preparation), Anatomy, UNSW, Prof. Michael Perry, Physiology and Pharmacology, UNSW, Dr Malcolm Handel, Medicine, UNSW, Prof. Charles Mackay, Garvan Institute, Dr Garry Corthals and more recently, Prof Greg Cooney, Garvan Institute.

(e) Local, national and international profile

Since 1992 I have given several invited presentations at local institutions including the Department of Clinical Pharmacology, St Vincent's Hospital (annually since 1992), the Heart Research Institute, Camperdown (1992 and 1996), the Centre for Immunology, St Vincent's Hospital, (1992 and 1999), Royal North Shore Hospital (1993 and 1996), St George Hospital (1993), the Department of Pharmacology, University of Sydney (1994 and 1998), the Department of Physiology and Pharmacology, UNSW (1994), the Rheumatology Department, St Vincent's Clinic (1994), the Department of Biochemistry, UNSW (1995), the Department of Pharmacy, University of Sydney (1997), the Victor Chang Cardiac Research Institute (1997), the Garvan Institute of Medical Research (1998), the Prince of Wales Hospital (1999), the Department of Rheumatology, Westmead

Hospital (1999). In the last five years I have been invited to speak at institutions including the Kolling Institute, the Oncology Research Centre, Prince of Wales Hospital.

I have been involved in local societies, organising and participating in the inaugural ASMR state scientific meeting in 1994 (Abstract 36) and acting as session chair and/or member of the scientific judging panel in each of the subsequent meetings. I have also played an active role in the local biochemistry community since 1992, serving as a member of Sydney Protein Group committee, a special interest group of the Australian Society for Biochemistry and Molecular Biology. I have made several presentations (Abstract 24-26, 40, 45, 48, 53, 64, 65) at other local meetings notably an invited lecture in the annual St Vincent's Symposium series (Abstract 39).

My national profile is reflected in invitations to speak in symposia at various National Meetings, commencing with a symposium presentation at the Australian Society for Microbiology meeting, Canberra, in 1981 and a workshop on new techniques in molecular biology at the Lorne genome meeting, 1988. More recently I have been invited to speak at the Australian Society for Medical Research meeting (Brisbane, 1992), the Australian Animal Technicians Association meeting (Sydney, 1996), the inaugural "Commercialising Health Forum" meeting (Sydney, 1997) and the inaugural meeting of the Society for Leukocyte and Inflammation Research, held conjointly with the Australian Society for Immunology Meeting (Melbourne, 1998). I was invited to chair a symposium on symbiotic nitrogen fixation at the Australian Society for Microbiology meeting in 1988. From 1992 to 1999 I have acted as session chair and/or member of judging panels at each of the annual National Meetings of the Australian Society for Medical Research. Since 1996 I have made presentations (Abstracts 47, 62-64) and been involved as session chair in the biennial East Coast Protein Meeting, a combined meeting of the Sydney and Queensland protein groups held at Byron Bay. I have given invited presentations to the Institute of Veterinary and Medical Sciences, Adelaide, and several presentations to the Department of Obstetrics and Gynaecology, Royal Women's Hospital, Melbourne. I was also given an honorary position in that Department in 1993 in recognition of my collaborative contributions. I have also made oral and poster presentations at several national meetings from my own lab and in collaboration (Abstracts 11-18, 23, 35, 37, 38, 49, 54-57, 67, 70, 71).

My international profile is reflected in presentations at the 2nd International Symposium on Trauma, Shock and Sepsis, Munich, Germany, 1991 (Abstracts 20-22), two of which were chosen for publication in the proceedings of the meeting (conference proceedings 18,19). I received invitations to speak at the 8th International Congress on Prostaglandins and Related Compounds, Montreal, Canada (1994) (Abstract 28) and the 2nd World Congress on Inflammation, Brighton, U.K (Abstract 45). My work has also been selected for poster presentation at several international meetings, notably at the American College of Rheumatology meeting (Abstract 27, 58) Gordon Conference (Abstract 59) and three Keystone Symposia (Abstract 60, 69, 72), the most recent of which has been selected for an oral presentation. My collaborative work has been presented at several international meetings (Abstract 19, 29-34, 41-43, 51, 66). I have given invited lectures at the National Cancer Institute, Frederick, Washington, USA, the Wellesley Hospital, Toronto, Department of Molecular Biology and Biochemistry, University of California, Irvine, Department of Biochemistry, Massey University, New Zealand, DuPont Ltd, Boston, Boehringer Mannheim Ltd, Penzburg. I was also invited to attend a Roussel-UCLAF meeting on sepsis in Paris, 1992. My recent work has been the subject of invited presentations to the second international meeting on phospholipase A2 (Berlin, 2004), the 3rd International meeting on phospholipase A2 (Sorrento, 2007) and the 4th International conference on Phospholipase A_2 and lipid mediators, (Tokyo, 2009).

(f) Postgraduate and undergraduate teaching

Curriculum Vitae: Dr Kieran F. Scott (April, 2010).

Ph.D Student Cosupervision.

1983-1986 Dr. J.J. Weinman, Australian National University. "Structure Function Studies of Nitrogen Fixation Genes in Broad-Host-Range *Bradyrhizobium*".
Current position. Research Officer, RSBS, Australian National University.

1983-1986 Dr. S. M. Howitt, Australian National University. "Physiological and Metabolic Studies on Nitrogen Fixation in Broad-Host-Range *Bradyrhizobium*".
Current position, Senior Lecturer, Faculty of Science, Australian National University.

1985 - 1987 Dr. M. N. Upadhyaya, "Molecular Genetics of Leaf-curl Diseases in *Rhizobium*-legume Interactions".
Current position. Principal Research Scientist, CSIRO, Plant Industry, Canberra.

1989 -1994 Dr A. Tseng, "Structure-Function Studies on Human Secretory (Type II) Phospholipase A_2" Current position: unknown

1989-1994 Dr. R. Seymour, "Molecular Design of Antibody-Toxin Conjugates for the Treatment of Leukaemia".
Current position unknown.

1992 -2000 Dr. M. Bidgood, "Secretory phospholipase A_2-cyclooxygenase pathways in rheumatoid arthritis". Winner 1997 Campion-Ma Playoust Award, ASMR.
Thesis submitted February, 2000. Current position, Pharmacist

1995 - 2001 Ms. C. Salom. "Regulation of secretory phospholipase A_2 expression in human rheumatoid synovial fibroblasts". Did not complete. Current position: Director, Centre for Addiction Research and Education, DRUG ARM, Queensland.

1999-2003 Dr Megan Taberner. " Global gene expression in human rheumatoid synoviocytes".
Current Position: Senior Associate, Mitchell Madison Group, London UK.

2003-2007 Dr Lawrence Lee. "Inhibiting the multiple actions if human group IIA secreted phospholipase A_2" Post-doctoral Fellow, Victor Chang Cardiac Research Institute

Masters Cosupervision.

1995 – 2001 Mr. O. Jamal. "Expression of secretory phospholipase A_2-IIA in arthritic synovium". Did not complete.
2005. Cosupervisor Abdel Qader Masters of Biopharmaceutival Research, UNSW
2006. Cosupervisor, Vinod Kumar, Masters of Biopharmaceutival Research, UNSW

Cosupervision, undergraduate Honours Students.

1986 Dr. T. Holton. "Transcriptional Analysis of *nif* Gene Expression in *Rhizobium*.".
1987 Ms. J. Stanton. "Identification of Nodulation Genes in *Bradyrhizobium*."
Mr. P. Williamson, "Restriction Fragment Length Polymorphisms in Soybean (*Glycine max*.)"

1992	Ms. Louise Graham. "Phospholipase A_2 in Asthma".
2000	Ms Sally Robins "The effect of paracetamol on the prostaglandin pathway in human rheumatid synoviocytes"
2003	Mr Jeremy Keh "The effect of phenolic antipyretic and analgesic drugs and their metabolites on myeloperoxidase activity"
2004	Mr Chris Young. " The effect of reducing agents on paracetamol-mediated analgesic action in the mouse acetic-acid-induced writhing test.

Undergraduate Teaching.

1983	Lecturer in the Research School of Biological Sciences course "Molecular Genetics and Recombinant DNA technology"
1986	Invited Lecturer, Botany Department, ANU.

(g) Administrative responsibilities.

My most recent position carries the administrative responsibilities of a level C academic, particularly responsibility for the day-to-day administration of a research group including financial management of grants, preparation of research reports and provision of reports on student performance to higher degrees committees. I have also served on the St Vincent's campus seminar committee and the Garvan Institute library committee. Prior to that I was the Scientific Director of an R&D Syndicate with a budget of $1.03 million pa. over a 3 year period. I was responsible for all aspects of the management of this project. I had administrative responsiblilties for my research group during my time at Pac Bio and served on the research strategy committee of that company..

(h) Peer review involvement

From 1983 to 1989 I was a reviewer of grants for United States Department of Agriculture. I currently review 1-4 grants per year for each of the following agencies; the Australian National Health and Medical Research Council, the Australian Research Council, The Australian Rheumatology Association, the United Kingdom Arthritis and Rheumatism Council, the Anti-Cancer Council of Victoria and the Community Health and Anti-tuberculosis Association. I review 1-4 manuscripts per year for the Medical Journal of Australia, Inflammation Research, the Journal of Leukocyte Biology, the Journal of Immunology, the European Journal of Biochemistry, Immunopharmacology, the American Journal of Pathology, Biochimie. I have acted as PhD examiner on seven occasions for theses submitted to Massey University (New Zealand), the University of Melbourne, the University of Sydney and Monash University. I have served on judging panels for scientific prizes at several State and National meetings of the Australian Society for Medical Research, served on the judging panel for Sydney Protein Group travel scholarships over several years and the panel for the Thompson Prize for student presentations in 1999. I am currently on the editorial board of two review journals, "Recent Patents on Anticancer Drug Discovery" and "Recent Patents on Cardiovascular Drug Discovery" and one open access Journal "The Open Cancer Journal".

(i) Scientific discipline involvement

I hold membership in the American Association for the Advancement of Science and three national societies, the Australian Society for Medical Research (ASMR), The Australian Society for Biochemistry and Molecular Biology (ASBMB) and the Society for Lymphocyte and Inflammation

Research. I am a member of two special interest groups of ASBMB, the Sydney Protein Group and the Sydney Transcription Group.

From 1991 to the present I have been involved in committees of the Australian Society for Medical Research (ASMR), first as a member and Hon. Treasurer of the state branch. From 1993 to 1997 I was on the Board of ASMR serving on several committees and as member of the executive committee from 1995-1997. I served as President in 1997. I currently serve as chair of the Research Fund of ASMR. I have been on the committee of the Sydney Protein Group since 1995. I was also a member of the research committee of the New South Wales State Cancer Council in 1997 and 1998. This committee was responsible for providing research policy advice to the Cancer Council Board and for the administration of the Council's research grant scheme

I was a member of the organising committee of the Lorne Genome Conference in 1988 and convened the inaugural scientific meeting of the ASMR state branch in 1992. I served on the Local organising committee of the ASMR national meeting held in Sydney in 1993. As a member of the ASMR board, I was responsible for overseeing the National Scientific Meetings held in my state. I have organised two scientific meetings for the Sydney Protein Group with invited international speakers and two meetings featuring local speakers. Both State and National ASMR meetings generate strong media interest and I have had extensive experience in the coordination of both print and electronic media coverage for these scientific meetings using several public relations firms.

Through the public affairs function of ASMR, I have had extensive experience with the organisation of research awareness programmes, particularly ASMR's annual national public awareness campaign "Medical Research Week". I have been involved in all aspects of these campaigns including fund raising, planning, liason with contracted public relations firms, writing and recording community service announcements for radio and press releases for the print media. I have conducted numerous national and local radio interviews promoting the benefits of research and spoken at meetings of several community groups such as Rotary Clubs.

ASMR also has a major political function in representing the views and aspirations of the medical research community, acting as an umbrella organisation for some 35 professional societies. In my term as president, the ASMR played a significant role in resolving difficulties associated with proposed changes to the research policy and direction of the New South Wales State Cancer Council. I instigated negotiations with the New South Wales State Government which resulted in a redrafted Cancer Council Act which guaranteed representation of researchers actively involved in cancer research through an ASMR nominee on the Board. Also during my presidential term, I, along with the ASMR board devised and raised funds for a campaign to double the budget of the Australian National Health and Medical Research Council. This campaign was successfully concluded with the announcement of a doubling of funding for research in the 1999 Federal Budget. It is widely acknowledged that ASMR played a significant role in that outcome through its lobbying and public relations activity.

(j) Wider community involvement

In addition to my work with ASMR, I have been involved in a health awareness campaign with respect to arthritis, serving on the planning committee for this campaign run by St Vincent's Hospital. I presented a public lecture on prospects for arthritis treatment and the benefits of research during National Science Week, 1999 at the Garvan Institute. As part of this awareness campaign I have also conducted several radio interviews specifically related to arthritis research. I promote science and medical research at our local primary school through involvement in the P&C

organization and fundraising for an annual science fair and provision of science resources to support the curriculum. Most recently I have been an invited speaker at several fundraising events for the NSW Cancer Council "Relays for Life".

(k) Research grant support

1985 "Nodulation genes in Rhizobium". K. F. Scott, J. M. Watson, $5,000 CSIRO/ANU Research Grant

1986 "Nodulation genes in Rhizobium" K.F. Scott, J.M.Watson, $16,000, CSIRO/ANU Research Grant.

1986-1989 Team Leader's Grant National Research Fellowships, K.F.Scott, $82,500 ,ARC

1987 "Nodulation genes in Rhizobium", K.F.Scott, $4,500, J.M. Watson, CSIRO/ANU Research Grant

1993-1995 "Production of a Recombinant Immunotoxin with Potential Utility in Leukaemia Therapy", $43,000 p.a. A/Prof. K Atkinson, Prof. J. Biggs, Dr. P. Kearney, Dr. K. F. Scott. NHMRC.

1993-1995 "Phospholipase A_2 in Labour Onset". $100,000 p.a. Dr. G. Rice, Dr K.F. Scott, Prof. S. Brennecke and Prof. G. Thorburn. NHMRC.

1993 "Phospholipase A_2 in Arthritis". $6,000. Dr. K.F. Scott, Prof. P.M. Brooks. Arthritis Foundation of Australia Grant in Aid.

1993 "Structure-Function Studies on Secretory Phospholipase A_2". $12,000. Dr. K.F. Scott and Dr. P. Curmi. ARC Small Grant Scheme.

1993 "Phospholipase A_2 in Arthritis". $ 10,430. Dr. K.F Scott and Prof. P.M. Brooks. Rebecca Cooper Foundation.

1994 "Structure-Function Studies on Secretory Phospholipase A_2" Dr. K. F. Scott and Dr. P. Curmi. $12,000. ARC Small Grant Scheme.

1995-1997 "The Role of Human Type II Secretory Phospholipase A_2 in Rheumatoid Arthritis". Chief Investigators Dr K.F. Scott, Prof. P.M. Brooks. Associate Investigator Dr. A. Tseng. $103,000 p.a. NHMRC .

1996 "Macromolecular Crystallographic Analysis Facility". $815,000. Dr P. Curmi, Dr K. F. Scott, Dr S. Breit, Dr. B. Mabbutt. UNSW Capital Grant.

1996 "Transcriptional Control of Secretory Phospholipase A_2 gene expression". $10,000. Dr K.F. Scott, ARC Small Grant Scheme.

1996 "Molecular Modelling Equipment". $100,000. Prof. R.M. Graham, Dr P. Reik, Dr. D. Ogg, Dr B. Church, Dr K.F. Scott., Prof. P. Brooks, Dr T. Iismaa, Dr H. Herzog, Prof. J. Shine. Viertel Foundation.

1997 "Resources for Structural Biology". $250,000. Dr. P. Curmi, Dr K.F. Scott. UNSW Capital Grant.

1997 "Transcriptional Control of Secretory Phospholipase A_2 Gene Expression". $11,000. Dr K. F. Scott. Rebecca Cooper Foundation.

1998-2000 Modulation of Cytokine-mediated Inflammatory Responses by Human Type II Phospholipase A_2. $130,000 p.a. Dr K F Scott, Prof P Brooks. NHMRC.2000

2000 Glaxo-Wellcome: $35,000. Detection of transcripts encoding enzymes of the eicosanoid biosynthetic pathway in cells from human blister fluids. Dr K.F. Scott

2000-2001. Smithkline Beecham (Australia and UK) $70,000. The effect of paracetamol on prostaglandin production by human rheumatoid synovial cells in culture. Dr G.G. Graham and Dr K. F. Scott

2001-2002 GlaxoSmithkline (Australia and UK) $ 50,000 The effect of paracetamol on prostaglandin production by human rheumatoid synovial cells in culture. Dr G.G. Graham and Dr K. F. Scott

2001-2002. Rebecca Cooper Foundation. $15,000. Research on phospholipase A_2.

2003-2005 The mechanism of action of secreted phospholipase A_2 and its inhibition in inflammation. $175,000 p.a. Dr K.F. Scott, Prof G.G. Graham, A/Prof HP McNeil. NHMRC

2004-2006 Department of Veterans Affairs. Dong Q, Scott K. F., Graham G and Russell PJ: $490,500. Oncogenic action and therapeutic potential of PLA_2 in prostate cancer.

2007-2009. NSW Cancer Council. Scott K. F., Graham, G. G., Dong, Q., Russell, P. J., $300,000 Secreted phospholipase A_2 in prostate cancer.

(l) Other relevant data

My career to date has been focused on research problems that have potential commercial value. My Ph.D studies resulted in the filing of several patents in the USA and Europe, most of which have issued. In recent years I have had experience in the commercialisation of focused research being involved in both the management and execution of research as well as gaining experience with patent and other business issues relating to the specific projects for which I was responsible. At Pacific Biotechnology, I served on the project evaluation committee which evaluated development projects on both scientific and commercial grounds. I was responsible for a team of twelve researchers over a three year period. This work was funded by two successful Government grants (i) a Generic Technology Grant from the Department of Industry Technology and Commerce (DITAC) in 1988 entitled "Recombinant biosynthesis of multi-subunit proteins requiring post-translational modification for biological activity". $1,004,025. Principal Investigator, Dr. K.F Scott. Associate Investigators, Dr. L. Lazarus, Dr. J. Shine, Dr. P Gray, Dr. M. Stuart. and (ii) a Discretionary Grant from DITAC entitled "Therapeutic monoclonal antibody for treatment of septic shock". $1,080,050. Project Leader, Dr. K.F.Scott, Principal Researchers Dr. A. Protter, Prof. P. Gray, Dr. I. Rajkovic.

In addition to presentations at local and international scientific meetings, I have prepared and made business and scientific presentations to many multi-national biopharmaceutical companies including DuPont (Delaware), Johnson & Johnson (San Diego and New Jersey), Scios-Nova (San Francisco) and Serono (Geneva).

From 1995-1997 I managed a government-sponsored R&D Syndicate aimed at developing phospholipase A_2 inhibitors. Principal Investigators, Dr K. F. Scott, Prof. P. Brooks and Prof. J. Shine. $1.03M p.a. This gave me additional experience in the design and management of R&D projects and resulted in the filing of several patents. These funds also provided the necessary capital equipment for the establishment of a laboratory. My profile in industry was recognised by my appointment to the Executive Committee and as Chair of the Programme Committee of the 1st Commercialising Health Forum, a biennial international meeting held in Australia with a view to showcasing the Australian Medical Biotechnology Industry internationally.

Issued Patents.

1. U.S. Patent No: 5,001,061 Method utilising *nifD* promoter regions of *Bradyrhizobium japonicum* and *B.* (sp. *Parasponia*) useful for expressing foreign genes in root nodules.
2. U.S. Patent No: 5,008,194 Recombinant DNA molecules, plasmids, transformed bacteria, which have nitrogen fixation gene H promoter sequence from *Bradyrhizobium* sp. and foreign structural gene.
3. U.S. Patent No: 5, 045, 461; Method for increasing yield and nodulation by *Bradyrhizobium.*
4. U.S. Patent No: 5,137,816; Rhizobial diagnostic probes and *Rhizobium trifoli*i *nif*H promoters
5. U.S. Patent No: 5,484,718; Nodulation gene promoters
6. U.S. Patent No: 5,656,602; PLA_2 inhibitory compounds
7. Australian Patent No: 8823915 Recombinant DNA molecules for plants and bacteria containing promoter for a *nifH* gene of *Rhizobium trifolii* and a foreign structural gene.
8. European Patent No. 130,047. Bacterial strain containing recombinant DNA fragments especially in *Rhizobium* strains for improved nitrogen fixation.
9. Australian Patent No 668513 - PLA_2 Inhibitory Compounds.
10. European patent No 92914800.5, PLA_2 Inhibitory Compounds.
11. Australian Patent No 2003229143. "Method of inhibiting prostate cancer cell proliferation".

Pending patents:

1. PCT/AU03/00719 'Method of inhibiting prostate cancer cell proliferation". This patent is currently in National phases of examination in the United States, Canada, Europe and Japan.

Filed/lapsed patents

1. U.S. Patent Application No: 662,611, Repetitive sequences in *Rhizobium.*
2. U.S. Patent Application No: 875, 296, *Bradyrhizobium* (sp. parasponia) nodulation regulatory protein and gene.
3. U.S. Patent Application No 09/269402- Inhibitors of PLA_2
4. Australian Provisonal patent application "A novel enzyme"
5. International Patent application No PCT/AU99/00087 Cyclic peptide inhibitors of PLA_2

14. Publications.

Curriculum Vitae: Dr Kieran F. Scott (April, 2010).

Refereed journal articles

1. **Scott, K.F.**, Rolfe, B.G., Shine, J. (1981) Biological nitrogen fixation: Primary structure of the *Klebsiella pneumoniae nif* H and *nif* D genes. J. Mol. Appl. Genet. 1:71-81 (5 citations).

2. Cen, Y., Bender, G.L., Trinick, M.J., Morrison, N.A., **Scott, K.F**., Gresshoff, P.M., Shine, J., Rolfe, B.G. (1982) Transposon mutagenesis in rhizobia which can nodule both legumes and non legume *Parasponia*. Appl. Env. Microbiol. 43: 233-236. (27 citations)

3. Rolfe, B.G., Shine, J., Gresshoff, P.M., **Scott, K.F.**, Djordjevic, M.A., Cen, Y., Hughes, J.E., Bender, G.L., Chakravorty, A., Zurkowoski, W., Watson, J.M., Badenoch-Jones, J., Morrison, N.A, Trinick, M.J. *Rhizobium* and the genetics of a controlled disease. Australian Microbiologist 3: 33-37. (citations not available)

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THE DEVELOPMENT OF ANDROGEN-INDEPENDENT PROSTATE CANCER

Brian J. Feldman and David Feldman

The normal prostate and early-stage prostate cancers depend on androgens for growth and survival, and androgen ablation therapy causes them to regress. Cancers that are not cured by surgery eventually become androgen independent, rendering anti-androgen therapy ineffective. But how does androgen independence arise? We predict that understanding the pathways that lead to the development of androgen-independent prostate cancer will pave the way to effective therapies for these, at present, untreatable cancers.

ACTIVATING DOMAIN
Region of steroid hormone receptors that enhances target gene transcription.

ZINC FINGER
Protein module in which conserved cysteine or histidine residues coordinate a zinc atom. Some zinc-finger regions bind specific DNA sequences; others are involved in protein–protein interactions.

HEAT-SHOCK PROTEINS (HSP)
Molecular chaperones that are induced during cellular stress. They help regulate cellular homeostasis and promote survival.

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Apart from skin cancer, prostate cancer is the most common form of cancer in men and the second leading cause of cancer deaths in men in the United States[1]. Initial treatment is usually prostatectomy or radiation to remove or destroy the cancerous cells that are still confined within the prostate capsule. However, many patients are not cured by this therapy and their cancer recurs, or they are diagnosed after the cancer has spread. Tumour growth is initially androgen dependent. Androgen ablation (BOX 1), the mainstay of therapy for progressive prostate cancer, causes regression of androgen-dependent tumours, as documented by the work of Huggins over 30 years ago[2]. However, many men eventually fail this therapy and die of recurrent androgen-independent prostate cancer (AIPC). AIPC is a lethal form of prostate cancer that progresses and metastasizes. At present, there is no effective therapy for it. There are several pathways by which AIPC can develop. These pathways provide insights into the mechanism of androgen action and schemes by which cancer cells subvert normal growth control and escape attempts to treat the cancer. Understanding the pathways that lead to AIPC is the first step towards developing therapies for this lethal form of prostate cancer.

Mechanism of androgen action

Why do prostate cancer cells normally need androgens to grow and survive? Prostate cancer growth depends on the ratio of cells proliferating to those dying. Androgens are the main regulator of this ratio by both stimulating proliferation and inhibiting apoptosis. So, prostate cancer depends on a crucial level of androgenic stimulation for growth and survival. Androgen ablation (BOX 1) causes cancer regression because without androgen, the rate of cell proliferation is lower and the rate of cell death is increased, leading to extinction of these cells[3].

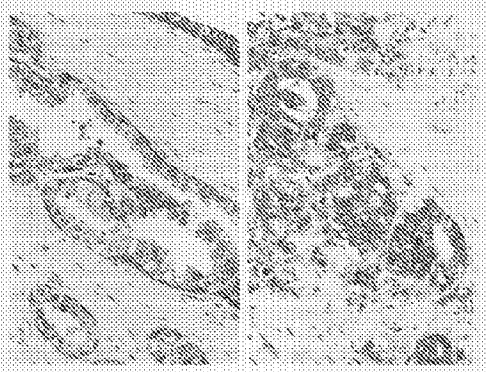
Testosterone — the main circulating androgen — is secreted primarily by the testes, but is also formed by peripheral conversion of adrenal steroids[4]. It circulates in the blood, where it is bound to albumin and sex-hormone-binding globulin (SHBG), with a small fraction dissolved freely in the serum. When free testosterone enters prostate cells (BOX 2), 90% is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (SRD5A2). DHT is the more active hormone, having fivefold higher affinity for the androgen receptor (AR) than does testosterone. The AR is a member of the steroid–thyroid–retinoid nuclear-receptor superfamily[5,6]. It is composed of an amino-terminal ACTIVATING DOMAIN, a carboxy-terminal ligand-binding domain and a DNA-binding domain in the mid-region that contains two ZINC FINGERS. Like other nuclear receptors, in the basal state, the AR is bound to HEAT-SHOCK PROTEINS and other proteins in a conformation that prevents DNA binding. Binding to

Box 1 | **Androgen ablation therapy**

More than 30 years ago, Charles Huggins showed that orchiectomy (removal of the testes) induced the regression of prostate cancer[2]. Since that time, androgen ablation has been the main therapeutic intervention for the treatment of hormone-sensitive prostate cancer[32]. The therapy is very effective in androgen-dependent cancer, but these cancers eventually become androgen independent, and go on to progress and metastasize. Although orchiectomy is an effective means of depleting androgens, pharmacological methods are now available. Gonadotropin-releasing hormone (GnRH) super-agonists (also referred to as luteinizing-hormone(LH)-releasing hormone analogues) downregulate the GnRH receptor in pituitary gonadotropes, leading to the suppression of LH release and inhibition of testosterone secretion from the testis[29]. GnRH antagonists are now in development that immediately antagonize LH release, avoiding the initial stimulation of testosterone secretion that occurs with GnRH super-agonists. Total androgen ablation[31], also referred to as maximal androgen blockade, combines an androgen receptor (AR) antagonist (anti-androgen) with a GnRH inhibitor. AR antagonists also prevent androgens produced by the adrenal glands from binding androgen receptors in the prostate. Total androgen ablation has not yet been shown to prolong survival[33], although it might be helpful in selected patients. It is unclear why the rational use of combination therapy does not improve survival compared with monotherapy, and further study is needed on this important therapeutic question[34]. Use of intermittent androgen ablation is being studied as a means of preventing or delaying the transition of cancer cells to androgen-independent prostate cancer[24], which eventually develops in most cases.

androgens induces a conformational change in the AR that leads to dissociation from the heat-shock proteins and receptor phosphorylation[6], in part mediated by protein kinase A[7]. The ligand-induced conformational change facilitates the formation of AR homodimer complexes that can then bind to ANDROGEN-RESPONSE ELEMENTS (AREs) in the promoter regions of target genes. The activated DNA-bound AR homodimer complex recruits co-regulatory proteins, co-activators or corepressors, to the AR complex. As in other nuclear receptors, the ligand-induced, activated conformation involves a shift in the position of helix 12 of the receptor to form a surface to which co-activators can bind. The co-activators allow interaction of the AR complex with the GENERAL TRANSCRIPTION APPARATUS to stimulate or inhibit target gene transcription[8] (FIG. 1). Many AR target genes have been identified[3], and additional ones are being discovered using cDNA microarray technology[9].

Box 2 | **The prostate**



The main function of the prostate is to produce seminal fluid. The prostate is made up of epithelial glands and a fibromuscular stroma. The glandular epithelium, which gives rise to prostate adenocarcinoma, has three types of cells: basal, luminal secretory and neuroendocrine. There are fewer basal cells and their function is not fully understood, although they secrete components of the basement membrane. A subset of the basal cells might be epithelial stem cells for the luminal epithelial cells[86]. The luminal cells secrete components of prostatic fluid, express the androgen receptor and secrete prostate-specific antigen (PSA) in an androgen-dependent manner. The stroma is composed of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerves and some infiltrating cells, such as mast cells and lymphocytes. Some stromal cells are androgen responsive and produce growth factors that act in a paracrine fashion on the epithelial cells. This stromal–epithelial crosstalk is an important regulator of the growth, development and hormonal responses of the prostate[93,94]. The well-organized secretory glandular structure (left) in the normal prostate, accentuated here by immunostaining for E-cadherin, becomes disrupted in invasive prostate cancer (right). (Images courtesy of John McNeal, Stanford University Medical Center, Stanford, California, USA.)

Mechanisms of AIPC development

What triggers the development of AIPC in the first place? Genetic modification is a crucial factor for tumour progression, and the development of AIPC is no exception[10]. But cells have powerful mechanisms that normally guard the genome from mutations. It is possible that, like many other cancers, prostate tumours initially select for genetic changes that increase the likelihood of subsequent mutations[10]. One hint that this process might be important in some prostate cancers comes from research on the phase II detoxification enzyme glutathione S-transferase π. This gene is expressed in normal prostatic epithelium. Here, it catalyses the intracellular detoxification of electrophilic compounds, including some carcinogens, but it is not expressed in more than 90% of prostate cancers owing to methylation of its promoter in a cancer-specific fashion[11]. This is thought to be one of the earliest and most common genomic alterations observed in sporadic prostate cancer. A general increase in the mutation rate would then increase the likelihood of a cell developing ensuing mutations ('multiple hits') that allow the prostate cancer cell to grow independently of androgen[12,13]. Although the necessity of a primary hit is an intriguing possibility, further research is needed to evaluate whether it truly is a prerequisite for the mutations that lead to the development of AIPC.

When in the evolution of advanced prostate cancer do the mutations occur that lead to AIPC? An early study led Cher *et al.*[23] to suggest that "untreated metastatic tumours contain the bulk of chromosomal alterations necessary for recurrence to occur during androgen deprivation", which indicated that mutations might be an early event that is independent of the selective pressure of androgen blockade[23].

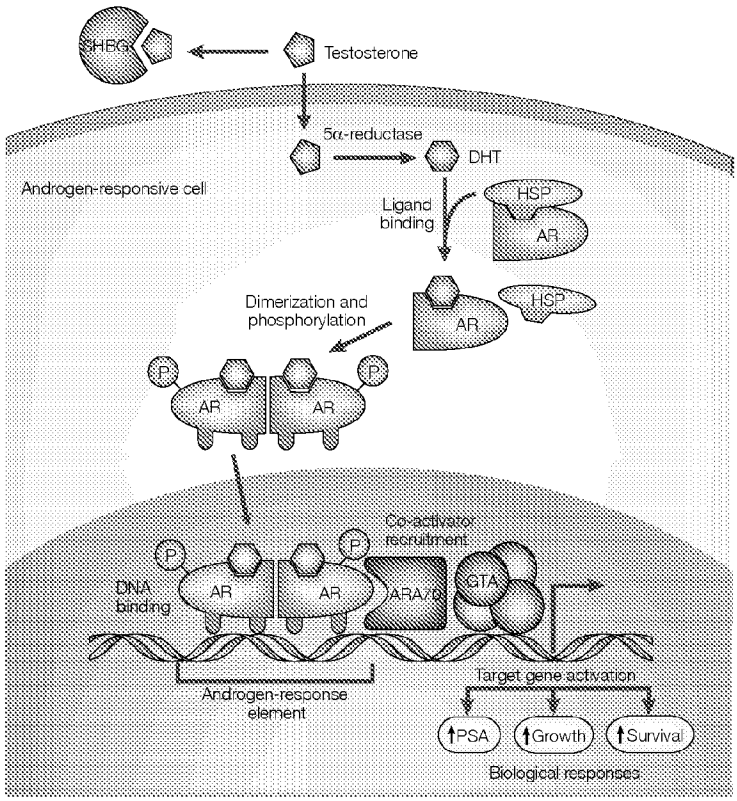


Figure 1 | **Androgen action.** Testosterone circulates in the blood bound to albumin (not shown) and sex-hormone-binding globulin (SHBG), and exchanges with free testosterone. Free testosterone enters prostate cells and is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. Binding of DHT to the androgen receptor (AR) induces dissociation from heat-shock proteins (HSPs) and receptor phosphorylation. The AR dimerizes and can bind to androgen-response elements in the promoter regions of target genes[6]. Co-activators (such as ARA70) and corepressors (not shown) also bind the AR complex, facilitating or preventing, respectively, its interaction with the general transcription apparatus (GTA). Activation (or repression) of target genes leads to biological responses including growth, survival and the production of prostate-specific antigen (PSA). Potential transcription-independent actions of androgens are not shown.

However, many studies have found only a few AR mutations in primary prostate cancer[14]; in comparison, metatastic prostate cancer frequently has mutations in the AR — possibly with a frequency as high as 50% (REFS 14–18). Mutations also might be common in other crucial pathways[10]. Recent investigations therefore support the theory that androgen ablation therapy provides selective pressure to target the androgen signalling pathway[16,18–20]. For example, therapy with the anti-androgen flutamide might select for mutant ARs in which flutamide acts as an agonist rather than an antagonist[18]. Even in the TRAMP (transgenic adenocarcinoma of mouse prostate) model of prostate cancer, in which SV40 large T antigen is overexpressed in the prostate luminal epithelial cells, mutations in the AR frequently develop, and different types of mutation are found in castrated versus intact mice[21,22]. So, the timing of the development of mutations that cause AIPC remains uncertain. Intermittent androgen ablation is considered a possible means of delaying the development of AIPC[24]. If treatment provides selective pressure for mutations that cause AIPC, intermittent treatment might reduce or delay the tendency towards development of mutant cells that become androgen independent. This important issue warrants further study.

The specific types of mutation that lead to AIPC will be discussed in the subsequent sections. We have categorized five potential mechanisms by which AIPC can develop (TABLE 1; FIG. 2). Some of these mechanisms also apply to other forms of steroid-hormone-independent cancer, such as breast cancer (BOX 3).

Type 1: the hypersensitive pathway

One possible mechanism by which a prostate cancer circumvents the effects of androgen ablation therapy is by increasing its sensitivity to very low levels of androgens. Prostate cancers that use this mechanism are not, strictly speaking, androgen independent — their responses still depend on AR and androgen — but they have a lowered threshold for androgens.

AR amplification. There are several potential mechanisms that would allow increased tumour-cell proliferation, despite low circulating androgens in the patient. One mechanism to accomplish this is by increasing the expression of the AR itself. Increased AR abundance leads to enhanced ligand-occupied receptor content, even in the face of reduced androgen concentration. Approximately 30% of tumours that become androgen independent after ablation therapy have amplified the AR gene, resulting in increased AR expression, whereas none of the primary tumours from the same patients before androgen ablation had an AR gene amplification[15,25]. These results indicate that amplification was probably the result of clonal selection of cells that could proliferate, despite very low levels of circulating androgens. Interestingly, patients with tumours that had AR amplification survived longer than patients with tumours that were refractory to ablation therapy but did not have amplification of the AR gene[15]. One possible explanation is that these amplified tumours are more differentiated than other prostate cancers, perhaps allowing the patients to have a better outcome.

Although tumours with AR amplification have increased levels of AR, the signal to proliferate presumably continues to require androgen[15,25]. This is an example of how tumours that seem clinically to be androgen independent could simply have increased their sensitivity to androgens so that they continue to proliferate in a low androgen environment. AR gene amplification that is detected in tumours that are progressing during androgen deprivation monotherapy with gonadotropin-releasing hormone (GnRH) analogues (BOX 1) might be associated with a favourable treatment response to second-line combined total androgen ablation with added anti-androgens[26]. This finding indicates that at least some AR-amplified tumours retain a high degree of dependency on residual androgens that remain in serum after monotherapy[26].

ANDROGEN RESPONSE ELEMENT (ARE). Site composed of hexanucleotide repeats and a spacer, usually in the promoter regions of target genes, that contains the androgen receptor zinc-finger-binding region.

GENERAL TRANSCRIPTION APPARATUS (GTA). A complex of proteins with the potential to facilitate transcription of genes. *In vivo* specificity of gene transcription by the GTA is regulated by interacting transcription factors.

Table 1 | **Mechanisms of development of AIPC**

Type	Pathway	Ligand dependence	AR dependence	Mechanism
1	Hypersensitive AR	Androgen dependent	AR dependent	Amplified AR Sensitive AR Increased DHT
2	Promiscuous AR	Pseudo-androgens Androgen antagonists Corticosteroids Coregulator mutations	Dependent on a mutant AR in LNCaP cells and AR^{cor} cells	Widened AR specificity Illicit stimulation by non-androgens 'Flutamide withdrawal' (antagonists acting as agonists)
3	Outlaw AR	Androgen independent Ligand independent	AR dependent	Mutant *PTEN* Amplified *HER-2/neu* Activated PI3K Activated MAPK Mutant coregulators
4	Bypass AR	Androgen independent	AR independent	Parallel or alternative survival pathways: • Overexpression of *BCL2* • Activation of other oncogenes • Inactivation of tumour suppressor genes
5	Lurker cells	Androgen independent	AR independent	Malignant epithelial stem cells

AR, androgen receptor; AR^{cor}, cortisol and cortisone responsive AR; DHT, dihydrotestosterone; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase.

Increased AR sensitivity. A second hypersensitive mechanism for tumour progression was found in animal models of the transition from androgen-dependent prostate cancer to apparent AIPC[27]. This pathway results in high-level expression of the AR, increased stability, and enhanced nuclear localization of AR in recurrent tumour cells. The tumour cells were also hypersensitive to the growth-promoting effects of DHT: the concentration of DHT required for growth stimulation in these AIPC cells was four orders of magnitude lower than that required for androgen-dependent LNCaP CELLS. These results indicate that the AR is transcriptionally active in some models of recurrent prostate cancer and can increase cell proliferation at the low circulating levels of androgen reported in castrated men[27].

Of course, it is also possible that some tumours that contain increased or amplified AR are not merely susceptible to low circulating androgens, but also have constitutive AR activation as described below (see outlaw receptors). Alternatively, tumours might also have amplified levels of co-activators[28], which could facilitate the induction of AR transactivation either by less active adrenal androgens or by lower levels of androgens.

Increased androgen levels. A third hypersensitive mechanism to circumvent androgen ablation therapy is by increasing the local production of androgens, to compensate for the overall decline in circulating testosterone. Prostate cells could increase the rate of conversion of testosterone to the more potent hormone DHT by increasing 5α-reductase activity. This would facilitate continued AR signalling even with significantly lower levels of serum testosterone. In support of this mechanism is the finding that, after androgen ablation therapy, serum testosterone levels decrease by 95%, but the concentration of DHT in prostate tissue is reduced by only 60% (REF. 29). Also, epidemiological studies have shown that certain ethnic groups who have higher levels of 5α-reductase activity have a higher incidence of prostate cancer[30]. Although the frequency of prostate cancer foci is similar in men from different ethnic groups, the proportion who develop clinically apparent cancer is higher in men of African descent than in Caucasians or men of Asian descent[10]. Men of African descent, who have a particularly high rate of prostate cancer, show the highest incidence of a polymorphism in the gene for 5α-reductase. This polymorphism substitutes a valine at codon 89 with a leucine (V89L), and results in significantly higher 5α-reductase enzyme activity. Men of Asian descent, who are at low risk for prostate cancer, have a low incidence of this polymorphism; men of Central- and South-American descent have both an intermediate incidence of the polymorphism and an intermediate risk[30]. In addition to genetic predisposition, it is also possible that, by selection during therapy, tumour cells either acquire mutations in the gene for 5α-reductase or select for increased expression of the enzyme. However, to our knowledge this has not yet been shown to occur in prostate cancer.

Recognition that patients can fail hormone ablation therapy even when very low serum levels of androgens are achieved led to the hypothesis that peripheral conversion of adrenal steroids to potent androgens could be sufficient to sustain the androgen signal, causing tumour growth and failure of androgen ablation therapy[31]. This hypothesis resulted in clinical trials using total androgen ablation (also called maximum androgen blockade) (BOX 1) to block residual androgen action at the AR[31]. However, so far, this therapy does not seem to provide any survival advantage[32–34].

LNCaP CELLS
A widely studied metastatic prostate cancer cell line that is androgen responsive.

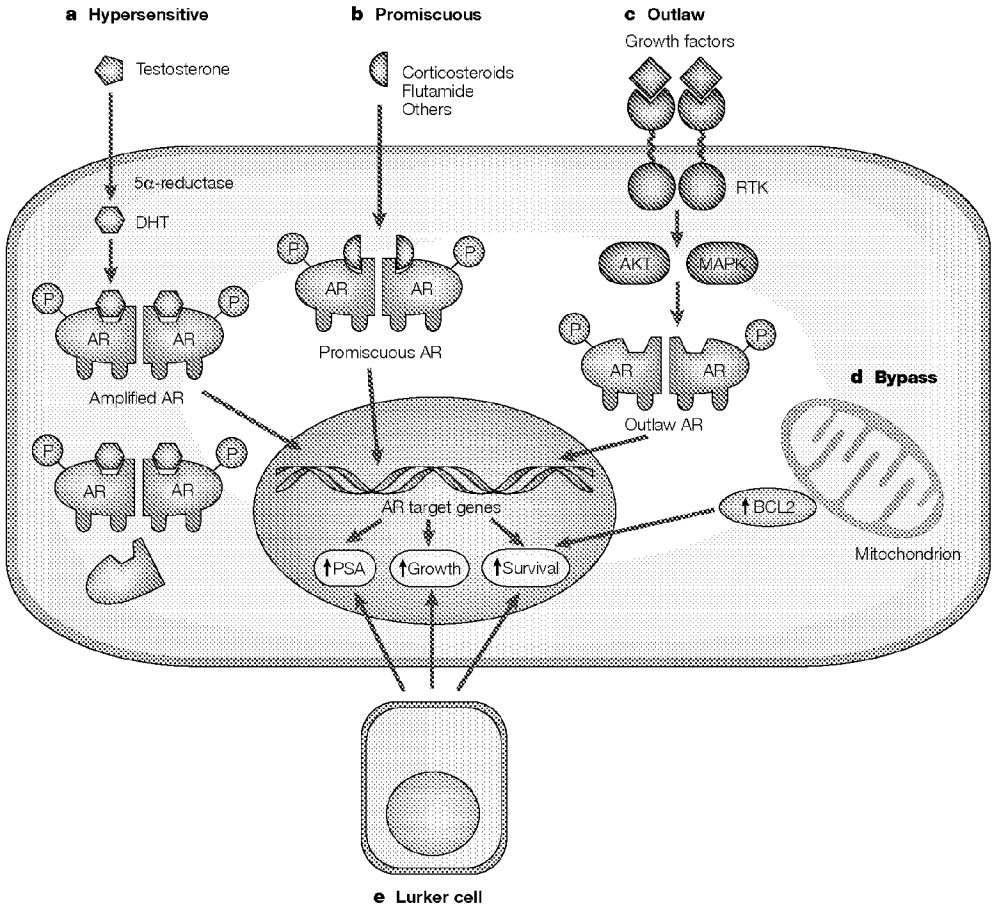


Figure 2 | **Five possible pathways to androgen independence. a** | In the hypersensitive pathway, more androgen receptor (AR) is produced (usually by gene amplification), or AR has enhanced sensitivity (not shown) to compensate for low levels of androgen, or more testosterone is converted to the more potent androgen, dihydrotestosterone (DHT), by 5α-reductase. **b** | In the promiscuous pathway, the specificity of the AR is broadened so that it can be activated by non-androgenic molecules normally present in the circulation. **c** | In the outlaw pathway, receptor tyrosine kinases (RTKs) are activated, and the AR is phosphorylated by either the AKT (protein kinase B) or the mitogen-activated protein kinase (MAPK) pathway, producing a ligand-independent AR. **d** | In the bypass pathway, parallel survival pathways, such as that involving the anti-apoptotic protein BCL2 (B-cell lymphoma 2), obviate the need for AR or its ligand. Finally, **e** | in the lurker cell pathway, androgen-independent cancer cells that are present all the time in the prostate — possibly epithelial stem cells — might be selected for by therapy.

Type 2: the promiscuous pathway

Most AIPCs express the AR protein. Whereas some of these tumours, at least initially, have adapted to the low-androgen environment, others acquire mutations that allow them to circumvent the normal growth regulation by androgens. It seems that many cases of AIPC do not develop from a loss of androgen signalling, but rather from the acquisition of genetic changes that lead to aberrant activation of the androgen signalling axis[21]. These changes are usually missense mutations in the AR gene that decrease the specificity of ligand binding and allow inappropriate activation by various non-androgen steroids and androgen antagonists.

AR mutations. The AR gene is located on the X chromosome and is not necessary for survival, so germ-line loss-of-function mutations in the AR, resulting in the androgen-insensitivity syndrome, are frequent[5]. The incidence of somatic AR mutations within prostate cancer cells is unclear owing to contradictory reports[14]. This is probably due to cellular heterogeneity within tumours, differences in the methodology for detecting mutations, and variations in the stage of tumours examined. Recent results, however, indicate that there is an increased incidence of somatic AR mutations in metastatic samples[14], confirming the earlier data of Taplin *et al.*[16]. Microdissection of tumours and laser capture techniques[35] will probably resolve this controversy, and this method should be considered in all future studies of AR mutations in metastatic specimens. On balance, it seems likely that the frequency of mutations in the AR is significantly increased in tumours after androgen ablation therapy, whereas most studies have reported few AR mutations in primary tumour samples collected before therapy[14,16,17]. This indicates that acquisition of mutations in the AR is likely to be

Box 3 | **Shared features of breast and prostate cancer**

The study of how androgen-independent prostate cancer (AIPC) develops raises interesting basic scientific questions about cancer biology, and there are many parallels with the development of steroid hormone independence in other tumours. The cancer that has provided the most insight into AIPC is breast cancer. Most important is the hormone-dependent nature of these cancers, leading to an interplay between the cancer cell and the endocrine system. In both types of cancer, this crosstalk has resulted in the use of endocrine modulators for therapy. Unfortunately, both cancers can progress to hormone-independent disease. As with prostate cancer cells and androgen receptors, breast cancer cells that express oestrogen receptors are dependent on oestrogens to promote proliferation and inhibit apoptosis. Effective breast cancer therapy in these tumours includes blocking the oestrogen receptor pathway using oestrogen antagonists (anti-oestrogens). The two anti-oestrogens in general use — tamoxifen and raloxifene — both have differential agonist and antagonist activity in various organs and are therefore called selective oestrogen receptor modulators (SERMs)[95]. A subset of patients treated with anti-oestrogens or SERMs will initially respond, but might later recur with oestrogen-independent tumours.

one mechanism for the development of AIPC. It seems reasonable that gain-of-function mutations that lead to a growth advantage by the tumour would be selected for. It is interesting that the loss-of-function mutations in the androgen-insensitivity syndrome are at different positions within the AR than the gain-of-function mutations found in prostate cancer[21].

Although only a few mutations in the AR have been studied in detail, a mechanism for the development of AIPC has emerged from these studies. In cells with these AR mutations, the androgen signal is maintained by broadening the number of ligands that can bind to and activate the receptor. Normally, the AR is specifically activated by testosterone and DHT, but mutations in the ligand-binding domain widen this stringent specificity. As a result, the malignant cells can continue to proliferate and avoid apoptosis by using other circulating steroid hormones as substitute androgens when the level of androgens is low.

The first AR mutation of this type was discovered in LNCaP cells[36]. LNCaP cells express high levels of AR, and androgens stimulate them to grow and express PROSTATE-SPECIFIC ANTIGEN (PSA) — a widely used and clinically important marker for prostate cancer cells. However, owing to a mutation in the AR, other steroid hormones, as well as the androgen antagonist flutamide, activate the AR and stimulate proliferation. Sequencing of the AR gene from LNCaP cells revealed a missense mutation in amino acid 877, which is located in the ligand-binding domain. This mutation results in the substitution of alanine for threonine at position 877 (T877A)[36] (FIG. 3). Molecular studies showed that hormones such as progestins, oestrogens and anti-androgens illicitly bind to this mutant AR and act as agonists[36]. During androgen ablation therapy, it is likely that this mutation undergoes clonal selection, conferring a growth advantage to cells that harbour the mutation[18]. Gaddipati *et al.*[37] examined 24 tumour samples from patients with metastatic prostate cancer and found the T877A mutation in six of the samples (25%), indicating that the mutation is relatively common in patients with AIPC. Promiscuous AR activators include adrenal androgens and metabolic products of DHT[19,38].

PROSTATE-SPECIFIC ANTIGEN (PSA). A serine protease in the kallikrein gene family that is secreted into seminal fluid by prostatic epithelial cells and found in the serum. As it is almost exclusively a product of prostate cells, measurement in blood has proved to be exceptionally useful as a tumour marker for diagnosis of prostate cancer and monitoring the effectiveness of treatment.

The promiscuous receptor mechanism can also explain the clinically observed phenomenon of 'flutamide withdrawal syndrome', in which patients show clinical worsening with flutamide, but then improve when flutamide is withdrawn[39]. Flutamide is an effective antagonist of the wild-type AR and so is used in androgen ablation therapy (BOX 1), but some patients treated with this anti-androgen experience a rapidly rising PSA level. This seems to be due to selection of AR mutations that yield a promiscuous receptor. In a series of bone marrow metastases, T877A mutations were found in 5 out of 16 patients who received combined androgen blockade with flutamide[18]. Cells harbouring these mutant ARs were strongly stimulated to grow by flutamide, whereas patients not treated with flutamide had different mutations that were not stimulated to grow by flutamide. These findings indicate that AR mutations occur in response to strong selective pressure from flutamide treatment[18]. In patients harbouring such tumours, discontinuing flutamide results in initial tumour regression before growth eventually resumes. On a molecular level, the T877A mutation changes the AR response to flutamide from an antagonist to an agonist. Interestingly, the T877A mutant AR does not have the same response to other anti-androgens such as bicalutamide (casodex). Many other mutations in the AR have been identified[40] and are catalogued in the Androgen Receptor Gene Mutations Database. It is unclear how many other mutations use the same promiscuous receptor mechanism and allow prostate cancer cells to become androgen independent.

Crystallographic studies of the ligand-binding domain of the wild-type AR[41] and the T877A mutant AR[42] have recently revealed that substituting alanine for threonine in the ligand-binding pocket explains the ability of the mutant AR to accommodate progesterone and other ligands that the wild-type receptor cannot. Similarly, the CWR22 tumour cell line has an H874Y mutation (substituting tyrosine for histidine) that influences binding of co-activator proteins by affecting the conformation of helix 12 (REF. 43).

The MDA PCa 2a and 2b cell lines, established from a bone metastasis in a patient who had recurrent metastatic disease that developed after orchiectomy[44], also harbour promiscuous ARs[45,46]. Like LNCaP cells, MDA PCa 2a and 2b cells express the AR, and androgen stimulates PSA expression and cell growth. However, the AR has reduced affinity for androgens, and MDA cells are less sensitive to androgens than LNCaP cells[46]. We identified two distinct missense mutations in the AR ligand-binding domain[46]. Double mutations in the AR have been reported previously[40], but never both in the ligand-binding domain. This mutated AR had the T877A mutation, as well as a previously identified[47] leucine-to-histidine substitution at amino acid 701 (L701H)[46]. We reasoned that these two mutations in the ligand-binding domain were likely to change the specificity of ligand binding to the AR (FIG. 3). In fact, the L701H mutation alone decreases the ability of AR to bind and respond to DHT[45]. However, the L701H

mutation also enhances the binding of other adrenal corticosteroids, particularly the glucocorticoids cortisol and cortisone. The T877A mutation has a synergistic effect by increasing the affinity of the AR for glucocorticoids by 300% more than the L701H mutation alone[45]. In this doubly mutated AR, cortisol and cortisone function as AR agonists (hence this mutant is named AR^{ccr}, for cortisol and cortisone responsive), and illicit binding leads to the induction of *AR*-responsive genes such as PSA (FIG. 3). So, in cells with the AR^{ccr} mutation, glucocorticoids can substitute for androgens and promote androgen-independent growth[45]. Because of the high affinity of glucocorticoids for the AR^{ccr}, it is likely that physiological levels of circulating cortisol and cortisone would be sufficient to promote tumour growth in patients with this double mutation. The frequency of this mutation in prostate cancer patients is unknown but the L701H mutation, which is sufficient to render the AR responsive to corticosteroids, has been reported three times[45]. Obviously, this type of patient should not be treated with hydrocortisone. However, some synthetic glucocorticoids have low affinity for the AR^{ccr} and might be useful therapeutically to suppress endogenous corticosteroids[45]. This hypothesis will require further investigation.

Recently, spontaneously occurring AR mutations have been found in the TRAMP transgenic model of prostate cancer[21,22,48]. These differ depending on whether the mice have been castrated. The mutations cluster in three regions of the AR: the highly conserved signature loop in all nuclear receptors; the region flanking the site where p160 CO-ACTIVATORS bind; and the boundary between the hinge and the ligand-binding domain. Consistent with the AR mutations described above, many of the AR mutations found in clinical cancer cases result in decreased specificity of ligand binding and inappropriate receptor activation by non-androgens, yielding a promiscuous AR phenotype[21]. But not all AIPCs with apparently promiscuous ARs harbour mutations in the AR. This has been shown by selecting for AIPC cells in castrated immunodeficient mice[49]. In another example, the LNCaP cell line was continuously selected for by growth in androgen-depleted medium over many passages, and a variant cell line emerged[50]. This cell line — called LNCaP-abl — had a fourfold higher expression of AR and a 30-fold increase in basal AR transcriptional activity compared with the parental LNCaP line. In the LNCaP-abl cell line, casodex which functions as an AR antagonist in the parental cell line — functions as an agonist[50]. But despite these changes, the AR in LNCaP-abl cells was not amplified and had only the parental LNCaP T877A mutation. Clearly, a mechanism other than mutations in the AR is promoting tumour progression. In these examples of experimental selection for AIPC cells, both in castrated mice and in cultured cells, one possibility is that co-regulatory molecules that interact with and either enhance or repress the AR signal might be responsible for the increase in AR responsiveness[50].

p160 CO-ACTIVATORS
p160 co-activators are a family of ~ 160-kDa proteins that act as co-activators of nuclear receptors. SRC1 and TIF2 are members of this family.

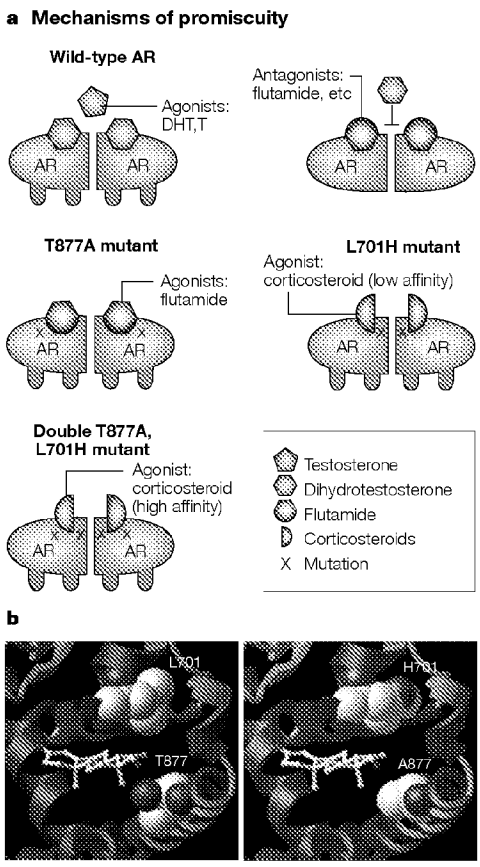


Figure 3 | **The promiscuous androgen receptor.** **a** | Mutations that broaden the specificity of the androgen receptor (AR): in the wild-type receptor, testosterone (T) and dihydrotestosterone (DHT) are agonists, whereas flutamide is an antagonist. The T877A mutant is activated by various non-androgenic steroid hormones, and flutamide also behaves as an agonist. The L701H mutant has reduced affinity for DHT and binds corticosteroids, but when the T877A and L701H mutations are combined, the resulting receptor (AR^{ccr}) has high affinity for corticosteroids. **b** | Models of the ligand-binding sites from the wild-type AR (left) and AR^{ccr} (right) with DHT bound, showing the extra space generated by the mutation of residues 701 and 887. Residues 701 and 877 are shown as space-filling models (carbon, white; nitrogen, blue; oxygen, red), and DHT is shown as a ball-and-stick model. A hydrogen bond can form between DHT and T877, but not between DHT and A877. (Images courtesy of Stanley R. Krystek and John Sack, Bristol-Myers Squibb, Princeton, New Jersey, USA.)

Co-regulator alterations. Several proteins act together with steroid hormone receptors as co-activators and corepressors of transcription[8]. A recent report of a case of androgen-insensitivity syndrome implicated an abnormal co-activator as the defect in androgen action, as the AR did not have a mutation[51]. Modulation of these co-regulatory proteins and their function is likely to be another mechanism by which prostate cancer progresses to AIPC. Breast and ovarian tumours — which can also progress from steroid hormone dependence to independence (BOX 3) — can use this mechanism. For

example, a member of the steroid receptor co-activator 1 (SRC1) family of nuclear receptor co-activators, AIB1, is amplified in some breast and ovarian tumours[52]. This protein interacts with the oestrogen receptor (ER) and enhances the transcription of oestrogen-regulated genes[52]. SRC1 family members seem to function in a relatively large number of tissue types, whereas a co-activator, ARA70, is said to be specific for androgen-responsive genes[53], although divergent results have been reported[54,55]. In the DU145 metastatic prostate cancer cell line, cotransfection of AR and ARA70 specifically enhanced transcription of androgen-responsive genes[56]. ARA70 also facilitates the conversion of several androgen antagonists to agonists in this cell line[56].

Gregory *et al.* recently showed that overexpression of two co-activators, TIF2 and SRC1, occurs in some specimens from recurrent prostate cancers and from prostate cancer cell lines. When combined with promiscuous ARs that have ligand-binding domain mutations, these changes are associated with increased AR activation, even at physiological concentrations of adrenal androgens[28]. The authors believe that most recurrent prostate cancers overexpress co-activators, thereby facilitating AR transactivation and enhancing responses to low levels of androgens. This would represent a combination of the hypersensitive pathway and the promiscuous pathway, and emphasizes the fact that several mechanisms can contribute to a single case of AIPC.

Although overexpression of co-activators is a possible mechanism for creating or enhancing promiscuous ARs in some tumours, a decrease in corepressor expression is equally likely to have similar effects. Again, research on breast cancer is a good model for this mechanism. Decreased expression of the nuclear receptor co-repressor (N-CoR) correlates with resistance to tamoxifen (BOX 3) in patients with breast cancer[57]. Normally, transactivation by the ER is blocked by tamoxifen if N-CoR is bound to the ER. Without the corepressor function of N-CoR, tamoxifen becomes an agonist, leading to activation of oestrogen-responsive genes[57]. Although there are, at present, no reports of similar events in prostate cancer, it is feasible that loss or decrease of AR corepressors would create a promiscuous AR by allowing molecules that normally do not activate the AR to take on the function of agonists. In the case of an androgen-ablated patient, corepressor loss might activate the AR signal for proliferation in the tumour cells, causing AIPC. Conformational changes in the AR, induced by various interacting proteins, are probably crucial for regulation of these events. Determining the crystal structure of these proteins will be vital for increasing our understanding of this mechanism of hormone-independent growth, as well as for developing effective treatment modalities.

Type 3: the outlaw pathway

Steroid hormone receptors that are activated by ligand-independent mechanisms have been referred to as 'outlaw' receptors[58]. An outlaw ER has been described in breast cancer, from which ERs with mutations that are capable of either dominant-positive or DOMINANT-NEGATIVE transactivation of oestrogen response elements were identified[58]. So far, no mutations in the AR have been reported to acquire this type of activity; however, other pathways can subvert the AR into becoming an outlaw.

Growth-factor-activated outlaw pathways. Certain growth factors, such as insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF), can activate the AR, creating an outlaw receptor, and can therefore induce AR target genes in the absence of androgen[59]. IGF-1, the most potent of the factors tested, induced a fivefold rise in PSA secretion in LNCaP cells[59]. These growth factors are ligands for receptor tyrosine kinases and initiate complex intracellular signalling cascades. It is unclear, at present, whether their effect on the AR pathway is direct or is the result of a downstream molecule that is induced in the signalling pathway. An intriguing supportive finding is the discovery that these growth factors seem to be overexpressed in some prostate cancers. Significantly, the AR antagonist casodex completely blocks activation of the AR by IGF-1, KGF and EGF[59]. This indicates that the AR ligand-binding domain is necessary for this outlaw activation. Furthermore, the ability of casodex to block IGF-induced AR activation makes this mechanism an unlikely explanation for AIPC in patients who fail casodex therapy. It is possible that upregulation of growth factor expression — combined with AR mutations — could result in AIPC in such patients, but further research is needed to test this hypothesis. Nevertheless, these experiments[59] had the important impact of highlighting the significance of tyrosine kinases in AR signalling and prostate cancer.

Receptor-tyrosine-kinase-activated outlaw pathways. Studies in breast and ovarian cancers have provided evidence of a connection between nuclear receptor signalling and receptor tyrosine kinases. HER-2/neu (also known as ERBB2) a member of the EGF-receptor family of receptor tyrosine kinases — is overexpressed in 20–30% of breast and ovarian cancers[60]. HER-2/neu has intrinsic tyrosine-kinase activity and can activate the ER in the absence of oestrogenic ligand. Therefore, overexpression of *HER-2/neu* could lead to oestrogen-independent stimulation of ER-mediated signal transduction pathways. Interestingly, in breast cancer, overexpression of *HER-2/neu* correlates with oestrogen independence[61], probably because HER-2/neu activation indirectly leads to phosphorylation and activation of the ER in the absence of oestrogen[62]. Phosphorylation therefore creates an outlaw ER, resulting in the oestrogen-independent growth of breast cancer cells[62].

The AR can be turned into an outlaw receptor by the same mechanism: *HER-2/neu* is consistently overexpressed in AIPC-cell sublines that are generated from XENOGRAFTS implanted in castrated mice[63], and androgen-dependent cell lines can be converted to androgen-independent cells by overexpressing *HER-2/neu*. Overexpression of *HER-2/neu* can activate AR-dependent genes in the absence of AR ligand[63,64], but not in the absence of AR. However, unlike the effect of IGF-1, the outlaw AR created by *HER-2/neu* overexpression could

DOMINANT NEGATIVE
A protein with an inhibitory signal that overrides or blocks a positive signal for transcription.

XENOGRAFT
A graft of tissue or cells transplanted between animals of different species.

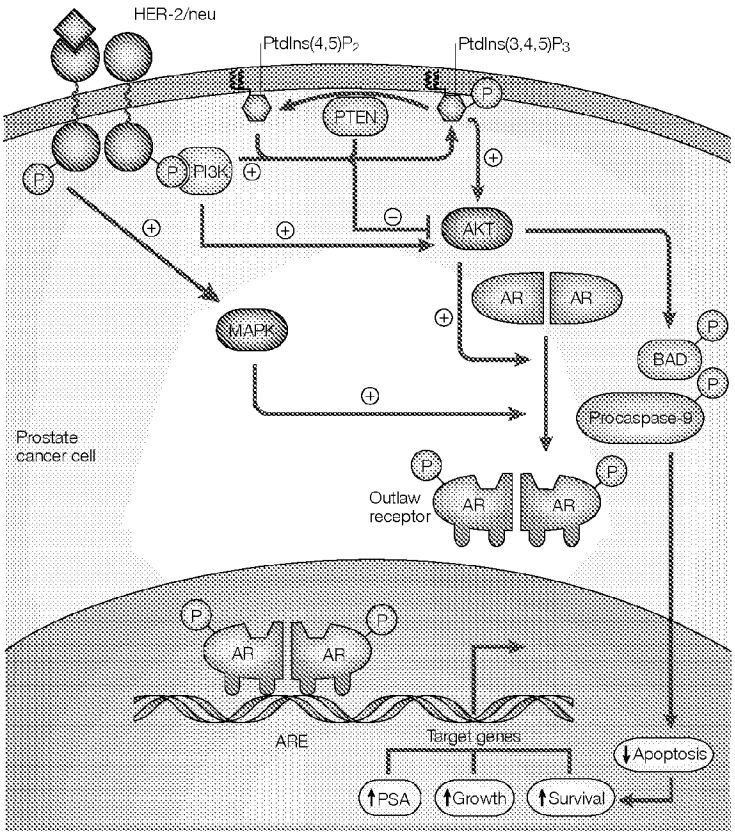


Figure 4 | **How growth factor signal transduction creates outlaw receptors.** In the tumour cells of a patient receiving androgen ablation therapy, HER-2/neu (and possibly other receptor tyrosine kinases) can become overexpressed. HER-2/neu indirectly activates mitogen-activated protein kinase (MAPK). MAPK might phosphorylate the androgen receptor (AR), creating an androgen-independent 'outlaw' receptor. An alternative means by which HER-2/neu (or other pathways) might activate the AR is by activating the AKT (protein kinase B) pathway. In this pathway, activation of receptor tyrosine kinases, such as HER-2/neu, increase the level of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) by activating phosphatidylinositol 3-kinase (PI3K). Another pathway might involve inactivation of the lipid phosphatase PTEN, so that PtdIns(3,4,5)P_3 can no longer be converted back to its substrate, PtdIns(4,5)P_2. AKT is activated by PtdIns(3,4,5)P_3, and might be able to produce an outlaw AR by phosphorylating it. AKT can also activate parallel survival pathways by phosphorylating and inactivating pro-apoptotic molecules such as BAD and procaspase-9. ARE, androgen response element; PSA, prostate-specific antigen.

not be blocked by casodex, indicating that this pathway is independent of the AR ligand-binding domain[63].

Taken together, these findings indicate that activation of HER-2/neu is an important mechanism for the progression to hormone-refractory disease in some breast and prostate cancers. This led to the therapeutic strategy of trying to prevent outlaw receptor formation by blocking the HER-2/neu receptor. Trastuzumab (Herceptin) — a monoclonal antibody against HER-2/neu — was developed as a therapeutic agent to block this pathway[65]. In patients with metastatic breast cancers that overexpress *HER-2/neu*, Herceptin increases the clinical benefit of first-line chemotherapeutic agents[65]. It also shows a benefit as a first-line agent in some patients who have failed other therapies[66]. Might Herceptin be of benefit in patients with AIPC? When tested in androgen-dependent (CWR22 and LNCaP) and androgen-independent (CWR22R) prostate cancer xenografts, Herceptin showed some antiproliferative activity in the androgen-dependent models but, when combined with the chemotherapy drug paclitaxel, it showed additive activity in both androgen-dependent and androgen-independent model systems[67].

Recent research has begun to reveal further details of the HER-2/neu signalling cascade in prostate cancer cells. Yeh *et al.* and colleagues[64] indicate that HER-2/neu could activate the AR through a mitogen-activated protein kinase (MAPK) pathway: inhibitors of MAPK decreased HER-2/neu-mediated activation of the AR. MAPK can phosphorylate the AR *in vitro*, and leads to AR activation in cell lines[64]. From these results, a hypothetical pathway for the development of AIPC can be predicted (FIG. 4). Although there is strong experimental evidence for this mechanism, future investigation is needed to ascertain whether this is truly a pathogenic pathway active in patients who develop AIPC.

The AKT pathway. Direct analysis of cancer samples has led to additional advances in our understanding of AIPC. An example of this was the discovery of the tumour suppressor gene *PTEN*, which was identified as a hot spot for mutations in glioblastoma, breast and prostate cancers[68], and is frequently, functionally inactivated in advanced metastatic prostate cancer[69]. PTEN is a lipid phosphatase that removes the 3-phosphate from 3-phosphorylated inositol lipids, such as phosphatidylinositol (3,4,5)-trisphosphate[70]. 3-phosphorylated inositol lipids are second messengers that activate a protein kinase called AKT or protein kinase B (PKB)[71–73] (FIG. 4). The AKT pathway has been suspected of contributing to tumorigenesis because of its anti-apoptotic activity. AKT phosphorylates and inactivates several proapoptotic proteins, including BAD and procaspase-9 (REF.74) (FIG. 4). So, in normal cells, by blocking the AKT pathway, PTEN allows cells to undergo apoptosis, whereas tumour cells that have lost PTEN function have increased AKT activity that blocks this signal for apoptosis. AKT has also been shown to regulate cell-cycle progression through a pathway that ultimately downregulates the cell cycle inhibitor p27 (REF.75).

Might the AKT pathway be involved in prostate tumour progression and the development of AIPC? To test this hypothesis, Graff and colleagues[76] established androgen-independent cell lines (LNAI) from xenografts of LNCaP cells that grew in castrated mice. They found increased AKT activity in the androgen-independent LNAI cell line compared with the parental androgen-dependent LNCaP cells. They also found that overexpressing AKT in LNCaP xenograft tumours accelerated tumour growth and downregulated the expression of p27 in these cells[76]. However, the aetiological role of AKT remains to be confirmed.

AKT might also be an alternative way by which HER-2/neu leads to outlaw AR activation[77,78] (FIG. 4), as HER-2/neu can activate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway[78]. AKT that has been activated by HER-2/neu signalling phosphorylates the

AR at serine (Ser) 213 and Ser791 (REF. 77), turning it into an androgen-independent outlaw receptor. Furthermore, this HER-2/neu-mediated activation of the AR could be blocked by expressing a dominant-negative AKT[77]. The relationship between these results and the activation of AR by the MAPK pathway are at present unclear, and whether this pathway is involved in AIPC development remains to be determined. However, *HER-2/neu* expression seems to increase with progression to AIPC[79], so no matter which of these kinases is responsible for the effect, therapeutic targeting of HER-2/neu in some cases of prostate cancer might be warranted. Recent investigation indicates that the AKT pathway might also be important in the development of tamoxifen-resistant breast cancer[80].

Type 4: the bypass pathway

The mechanisms discussed so far require the presence of the AR and its signalling cascade for the development of AIPC. However, it is also possible that complementary or alternative pathways can be invoked that are capable of bypassing the AR completely. As previously discussed, AR activation stimulates androgen-dependent cancer cells to proliferate, and depletion of androgens results in apoptosis. An effective bypass of the androgen signalling cascade would facilitate proliferation and inhibit apoptosis, even in the absence of androgens and AR. When crucial survival pathways are targeted by therapy, there might be selection for mutations that upregulate parallel pathways that can provide a substitute survival signal. In the case of prostate cancer patients being treated with androgen ablation, blocking the apoptosis signal would be one such pathway for tumour cell survival.

The *BCL2* gene is an obvious bypass candidate gene that can block apoptosis. *BCL2* is not normally expressed in the secretory epithelial cells of the prostate[81]. But *BCL2* is frequently expressed in pre-malignant PROSTATIC INTRAEPITHELIAL NEOPLASIA (PIN), as well as in AIPC[82]. Furthermore, Liu *et al.*[83] detected the emergence of *BCL2* expression in tumours that initially did not express it, by selecting for growth of prostate cancer xenografts in castrated mice. Blocking BCL2 with antisense oligonucleotides delayed the emergence of AIPC in a LNCaP xenograft model[84]. Upregulation of BCL2, then, could bypass the signal for apoptosis that is normally generated by androgen ablation. In support of this mechanism, many cases of AIPC, both in humans and in rodent models, have been found to overexpress *BCL2* (REFS 82,85). However, overexpression of *BCL2* is not essential for the formation of AIPC[85] — presumably because other bypass pathways or one of the other four mechanisms (TABLE 1) can substitute.

Further studies are needed to understand the exact mechanism by which these bypass pathways interact with AR signalling. It remains possible that the pathways directly intersect at a junction yet to be elucidated. Many other oncogenes and tumour suppressor genes, in addition to *BCL2*, could have a similar bypass role in the development of AIPC[10], but discussing each of these genes is beyond the scope of this review. It seems likely that androgen ablation therapy would provide the selective pressure needed for some tumours to adapt to and escape from the effect of therapy by invoking any of these bypass mechanisms.

Type 5: the lurker cell pathway

Androgen ablation fails because cells that are not dependent on androgen for growth take over and the tumour grows in an androgen-independent fashion. As this review has highlighted, there could be several mechanisms by which a cell can become androgen independent and so lead to failure of androgen ablation therapy. However, John Isaacs has postulated[86] that androgen ablation therapy might fail, and AIPC eventually develop, because a subpopulation of androgen-independent tumour cells was present even before therapy was initiated. The putative epithelial stem cells among the basal cells of the prostate are believed to be androgen independent: their rates of proliferation and death are not affected by androgen ablation[3]. According to this model[86], if the epithelial stem cell transformed and became the origin of a prostate cancer, the following events would occur: first, in the presence of androgens, most of the epithelial stem cell progeny would differentiate into androgen-dependent epithelial cancer cells that would comprise most of the tumour; second, after androgen ablation, the androgen-dependent cells would be eliminated but the androgen-independent malignant epithelial stem cells, which have been lurking in the background all along, would remain viable; and third, these malignant epithelial stem cells would continue to proliferate and ultimately result in the relapse of the disease as AIPC. It is tantalizing to consider that prostate tumours resist apoptosis and proliferate by adopting features of normal prostatic stem/progenitor cells, and that basal cells — the putative stem/progenitor cells of the prostate — are androgen independent, just like most advanced prostate cancers[87]. Craft *et al.*[20] provide evidence to support this hypothesis. They showed that the latter stage of androgen independence results from clonal expansion of androgen-independent cells that are present at a frequency of about 1 per 10^5–10^6 androgen-dependent cells. They conclude that prostate cancers contain heterogeneous mixtures of cells that vary in their dependence on androgen for growth and survival, and that treatment with anti-androgen therapy provides selective pressure that alters the relative frequency of these cells, thereby leading to outgrowths of androgen-independent cancers.

This hypothesis draws parallels with certain types of human leukaemia that relapse, despite effective therapy that had reduced the malignant cells to undetectable levels. This might occur because stem cells that are resistant to chemotherapy, lurking in the bone marrow, regenerate the malignant population[88,89]. The potential for a transformed prostate epithelial stem cell to produce androgen-dependent progeny needs further investigation.

PROSTATIC INTRAEPITHELIAL NEOPLASIA
(PIN). Dysplastic cellular changes confined to the prostatic epithelium and considered to be a precursor to adenocarcinoma of the prostate.

Concluding remarks

The study of the pathways by which AIPC develops has led to a fascinating overlap between the fields of endocrinology and oncology. The pathways show how malignant cells can hijack the endocrine system and develop alternative signalling pathways to subvert therapeutic attempts to control cell growth by androgen ablation. We do not believe that these five mechanisms exhaust the possibilities and, no doubt, further studies will reveal additional pathways. It is also possible, if not likely, that a single cancer uses several mechanisms either initially or in a multistep progression to AIPC. As prostate cancers use various schemes to subvert normal restraint on cell growth, successful therapy will require an individualistic approach based on the type of AIPC present. Effective therapy of AIPC will require that each patient's cancer be analysed so that a specific targeted therapy can be initiated[90]. To be successful, therapeutic measures will need to rescue the cells from the AIPC mechanism and restore normal growth regulation, or at least block the abnormal stimulation driving cell growth. Such approaches are already being developed[91], as exemplified by the use of Herceptin to treat breast and prostate cancers in which HER-2/neu hyperactivity is the cause of hormone independence[65]. By understanding the mechanisms exploited by the cancers, new therapeutic targets are being recognized[92]. We anticipate that fresh diagnostic measures and additional therapeutic options targeted at the specific defect will soon be added to our armamentarium in our efforts to thwart unregulated cancer cell growth.

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Online links

DATABASES

The following terms in this article are linked online to:

CancerNet: http://cancernet.nci.nih.gov/
prostate cancer | breast tumours | ovarian tumours

LocusLink: www.ncbi.nlm.nih.gov/LocusLink/
albumin | sex-hormone-binding globulin | 5α-reductase | androgen receptor | protein kinase A | glutathione S-transferase π | SRC1 | AIB1 | ARA70 | TIF2 | insulin-like growth-factor-1 | keratinocyte growth factor | epidermal growth factor | HER-2/neu | MAPK | *PTEN* | AKT | BAD | procaspase-9 | p27 | PI3K | *BCL2*

Medscape DrugInfo:
http://promini.medscape.com/drugdb/search.asp
flutamide | casodex | tamoxifen | Herceptin | paclitaxel

FURTHER INFORMATION

Androgen Receptor Gene Mutations Database:
www.mcgill.ca/androgendb

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Expression of Group IIA Secretory Phospholipase A2 Is Elevated in Prostatic Intraepithelial Neoplasia and Adenocarcinoma

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Phospholipase A2 (PLA2) enzymes release arachidonic acid from cellular phospholipids in a variety of mammalian tissues, including prostate. Group IIa secretory PLA2 (sPLA2) can generate arachidonate from cellular phospholipids. We examined the group IIa sPLA2 expression in benign prostatic tissues, prostatic intraepithelial neoplasia (PIN), and adenocarcinoma to determine whether sPLA2 expression is altered in the carcinogenesis of human prostatic cancer. Thirty-three of 74 total cases (45%) of benign prostatic tissue showed positive immunohistochemical staining for group IIA sPLA2, whereas 63 of 69 total cases (91%) of high-grade PINs and 70 of 78 total cases (90%) of adenocarcinomas gave positive results. Four of 10 cases of low-grade PIN showed positive immunoreactivity for sPLA2. The number of cells staining for sPLA2 was significantly less in benign epithelium (4%) and low-grade PIN (4%) compared to high-grade PIN (40%) or adenocarcinoma (38%) ($P < 0.001$). There was no significant difference between high-grade PIN and adenocarcinoma in the number of cells staining positively for sPLA2. The intensity of sPLA2 immunoreactivity was also different among benign prostatic tissue, low-grade PIN, high-grade PIN, and prostatic adenocarcinoma specimens. The malignant cells demonstrated more intense immunohistochemical staining (moderate to strong staining in 81% and 69% cases for high-grade PIN and adenocarcinoma, respectively) than benign glands (moderate staining in 11% of cases). No strong staining was observed in benign glands or low-grade PIN. Our data are consistent with the contention that group IIA sPLA2 expression is elevated in neoplastic prostatic tissue and support the hypothesis that dysregulation of sPLA2 may play a role in prostatic carcinogenesis.
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In mammalian cells, phospholipases A2 (PLA2s) are enzymes that release free fatty acids through catalysis of membrane phospholipids at the Sn-2 position. The resulting product, arachidonic acid, is metabolized to produce prostaglandins and leukotrienes that mediate a diverse array of biological activities including inflammation, mitogenesis, and tumor cell invasion. Several reports have implicated arachidonic acid and its metabolites as factors regulating cellular proliferation and apoptosis.[1–3] Arachidonic acid has also been implicated in the pathway of tumor necrosis factor- and Fas-induced apoptosis in various cell lines.[4–6]

Several types of PLA2s have been identified.[7,8] Seven low molecular weight (14 kd) sPLA2s (including group IB, IIA, IID, IIE, III, V, and X) are known to be present in both the intracellular compartment and extracellular milieu. The 40-kd form is a calcium-independent enzyme, which is referred to as iPLA2. Cytosolic PLA2 (cPLA2) is a high molecular weight (85 kd) form found predominantly within the cytosol of cells. cPLA2 activity is regulated by intracellular Ca^{2+} concentrations[8,9] and shows characteristic preference for hydrolyzing arachidonic acid at the sn-2 position. In contrast, sPLA2s have a broad substrate preference.[8]

Reports have been published linking arachidonic acid and its metabolites with prostatic malignancy.[10–15] Because phospholipase activity is required for phospholipid metabolism and subsequent generation of arachidonic acid, aberrant expression and function of sPLA2 may play a role in prostatic carcinogenesis. However, little is known about sPLA2 expression in human prostatic tissues. In this study, we determined the level of group IIA sPLA2 expression in specimens of human prostatic adenocarcinoma, its precursor lesion [(high-grade prostatic intraepithelial neoplasia PIN)], low-grade PIN, and benign prostatic tissue.

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Materials and Methods

Tissue Samples

Seventy-eight cases of radical retropubic prostatectomy and bilateral lymphadenectomy between 1990 and 1994 were obtained from the surgical pathology files of Indiana University Medical Center. Patients ranged in age from 51 to 78 years (mean, 63 years). Grading of the primary tumor from radical prostatectomy specimens was performed according to the Gleason system.[16] The Gleason grade ranged from 4 to 10. Pathological stage was performed according to the 1997 TNM (tumor, lymph nodes, and metastasis) system. Pathological stages were T2a (n = 11 patients), T2b (n = 35), T3a (n = 26), and T3b (n = 6). Six (8%) patients had lymph node metastasis at the time of surgery.

Generation of Rabbit Polyclonal Antibody to sPLA-IIA

Rabbit polyclonal antibody specific to sPLA2-IIA was generated by immunizing rabbits with purified, recombinant human sPLA2-IIA protein. The antisera were affinity-purified. The specificity of the purified IgG antibody was confirmed by staining Chinese hamster ovary (CHO) cells that stably expressed sPLA2-IIA. CHO cells expressing sPLA2-X or sPLA-V did not stain with this antibody (Eli Lilly and Company, Indianapolis, IN).

Immunohistochemical Studies

Serial 5-μm-thick sections of formalin-fixed slices of radical prostatectomy specimens were used for the studies. Tissue blocks that contained the maximum amount of tumor and highest Gleason grade were selected. One representative slide from each case was analyzed and we recognized the limitation of sample variation. Slides were deparaffinized in xylene twice for 5 minutes and rehydrated through graded ethanols to distilled water. Endogenous peroxidase activity was inactivated by incubation in 3% H_2O_2 for 15 minutes. The nonspecific binding sites were blocked by incubating with 10% normal horse serum in phosphate-buffered saline (PBS) (0.01 mol/L phosphate, ph7.4, 0.137 mol/L NaCl). Tissue sections were then incubated with the polyclonal rabbit antibody against human sPLA2 (1:76,000 dilution) for 60 minutes at room temperature. After washing with PBS, biotinylated goat anti-rabbit IgG was applied for 30 minutes. Additional washing was followed by incubation with peroxidase-labeled streptavidin for 30 minutes. Immunoreactivity was visualized by incubation of sections with diaminobenzidine in the presence of hydrogen peroxide. Sections were counterstained with light hematoxylin and mounted with a coverslip. All of the procedures were performed at room temperature. No enzymatic pretreatment was required for antigen retrieval. Positive and negative controls were run in parallel with each series and appropriate results were obtained.

The extent and intensity of staining were evaluated in benign epithelium, low-grade PIN, high-grade PIN, and adenocarcinoma from the same slide for each case. Microscopic fields with the highest degree of immunoreactivity were chosen for analysis. At least 1000 cells were analyzed in each case. The percentage of cells exhibiting staining in each case was evaluated semiquantitatively on a 5% incremental scale ranging from 0 to 95%. A numeric intensity score between 0 and 3 was assigned to each case on a scale from 0 to 3 (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining).

Statistical Analysis

The mean percentage of immunoreactive cells in benign epithelium, low-grade PIN, high-grade PIN, and adenocarcinoma were compared using one-way analysis of variance with a random subject effect to correlate the within-subject measurements. The intensity of staining in benign epithelium, low-grade PIN, high-grade PIN, and adenocarcinoma were compared using Cochran-Mantel-Haenszel tests for correlated ordered categorical outcomes. Pairwise comparisons between the tissue types were made if the analysis of variance revealed significant treatment effects. A P value <0.05 was considered significant, and all P values were two-sided.

Results

Immunoreactive group IIA sPLA2 was evident with an exclusive cytoplasmic staining pattern in cells (Figure 1). No immunoreactivity was seen in the stromal cells. sPLA2 immunoreactivity was found in 70 cases (90%) of cancer, 63 cases (91%) of high-grade PIN, 4 cases (40%) of low-grade PIN, and 33 cases (45%) of benign glands (Table 1). The number of cells staining in benign epithelium (mean, 3.8%) and low-grade PIN (mean, 3.5%) was much lower than in high-grade PIN (mean, 39.4%; $P < 0.0001$) or adenocarcinoma (mean, 37.5%; $P < 0.0001$) (Table 1). There was no significant difference in the percentage of cells staining positive for sPLA2 between high-grade PIN and adenocarcinoma or between benign epithelium and low-grade PIN. The percentage of malignant cell in adenocarcinoma staining for sPLA-IIA was divided into two categories according to the mean percentage of cell staining (<37% and >37% of cancer cells staining), and analyzed against the degree of tumor differentiation. The adenocarcinomas were grouped into low grade (Gleason grade 4 to 6), intermediate grade (Gleason grade 7), and high grade (Gleason grade 8 to 10). No statistically significant difference was identified between percentage of sPLA-IIA-positive cancer cells and Gleason grade (P = 0.59).

The intensity of staining also differed among benign epithelium, low-grade PIN, high-grade PIN, and adenocarcinoma (Table 2). The majority of benign glands (55%) and low-grade PIN (60%) did not show any reactivity. Moderate staining (intensity grade 2) was evident in 11% of the benign epithelium cases and no low-grade PIN cases. No benign epithelium or low-grade PIN showed

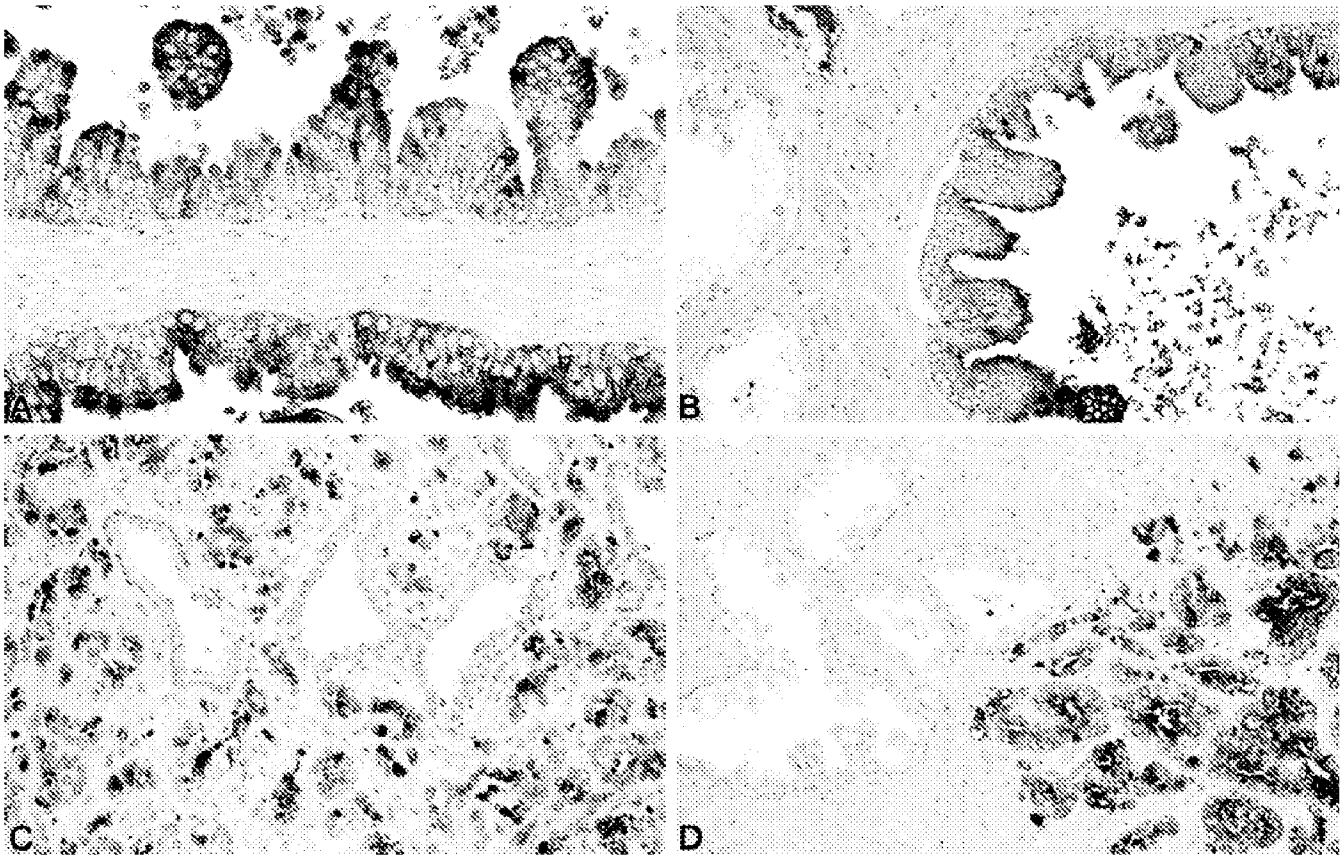

Figure 1. sPLA2 immunohistochemical staining of high-grade PIN (**A** and **B**) and prostatic adenocarcinoma (**C** and **D**). Immunoreactive sPLA2 was evident in malignant epithelial cells with a cytoplasmic staining pattern. In contrast, no or minimal immunoreactivity was seen in adjacent benign glands. For details, see the Materials and Methods section.

strong reactivity (intensity grade 3). In contrast, strong staining was evident in 29% (20 cases) of high-grade PIN and 33% (26 cases) of adenocarcinoma. Only a minority of the cases was negative (6% for high-grade PIN and 9% for cancer) or demonstrated weak staining (10% for high-grade PIN and 22% for cancer). The percentage of staining was statistically higher compared to that of the benign epithelium and low-grade PIN ($P < 0.0001$). There was no difference in intensity of cell staining between high-grade PIN and adenocarcinoma or between benign epithelium and low-grade PIN. The staining intensity of adenocarcinomas was also further analyzed according to the Gleason grade. No statistically significant difference was identified between staining intensity and Gleason grade ($P = 0.71$).

There was no significant correlation between the percentage of cells staining and patient age ($r = 0$), tumor stage ($r = 0.21$), lymph node metastasis ($r = 0.17$), Gleason grade ($r = 0.05$), or extent of tumor involvement in the prostatectomy specimens ($r = 0.16$).

Discussion

To our knowledge, this is the first report to characterize the immunohistochemical staining of group IIA sPLA2 in the high-grade PIN and prostatic adenocarcinoma. We found more intense cytoplasmic immunoreactivity for sPLA2 in neoplastic prostate tissues (high-grade PIN and adenocarcinoma) compared to benign glands and low-

Table 1. sPLA2 Immunoreactivity of Benign and Neoplastic Prostatic Tissues in Radical Prostatectomy Specimens

	No. cases	% of Cases staining	Mean % of cells staining ±SE	Range, %
Benign epithelium	74	45.2	3.8 ± 0.6	0 to 30
Low-grade PIN	10	40.0	3.5 ± 1.5	0 to 20
High-grade PIN	69	91.3	39.6 ± 2.9*	0 to 95
Adenocarcinoma	78	89.7	37.5 ± 3.8*	0 to 95

*, Indicates percentage of staining statistically higher compared to that of the benign epithelium and low-grade PIN with a P value < 0.0001 using analysis of variance. There was no difference in percentage of cell staining between high-grade PIN and adenocarcinoma or between benign epithelium and low-grade PIN.

Table 2. Intensity of sPLA2 Staining of Benign and Neoplastic Prostate in Radical Prostatectomy Specimens

	Staining intensity			
	0	1	2	3
Benign epithelium	40 (54.8%)	25 (34.2%)	8 (11.0%)	0 (0%)
Low-grade PIN	6 (60.0%)	4 (40.0%)	0 (0%)	0 (0%)
High-grade PIN*	6 (8.7%)	7 (10.1%)	36 (52.2%)	20 (28.9%)
Adenocarcinoma*	7 (9.0%)	17 (21.8%)	27 (34.6%)	27 (34.6%)

*, Indicates percentage of staining statistically higher compared to that of the benign epithelium and low-grade PIN using Cochran-Mantel-Haenszel tests ($P < 0.0001$ for benign epithelium and $P < 0.02$ for low-grade PIN). There was no difference in percentage of cell staining between high-grade PIN and adenocarcinoma or between benign epithelium and low-grade PIN.

grade PIN. The percentage of positively stained high-grade PIN and adenocarcinoma cells was significantly higher than observed in the benign prostatic glands and low-grade PIN. There were no significant differences in the extent or intensity of sPLA2 immunoreactivity between high-grade PIN and adenocarcinoma or between benign epithelium and low-grade PIN. Neither the extent nor the intensity of sPLA2 immunoreactivity in prostatic adenocarcinomas was related to Gleason grade.

Increased immunoreactivity for group IIA sPLA2 in prostatic neoplasia seen in the present study agrees with previously published observations by Faas and colleagues.[15] Using enzymatic analysis, these investigators demonstrated a two-fold enhancement of sPLA2 activity in human prostatic adenocarcinoma compared to benign prostatic tissue. Evidence indicates that malignant prostatic tissue contains significantly lower levels of arachidonic acid in phospholipids.[17,18] Altered phospholipid metabolism in malignant prostatic tissues may result from increased utilization of arachidonic acid for the formation of prostaglandins and eicosanoids. These arachidonic acid metabolites may be crucial for the growth and progression of malignant lesions. Chaudry and colleagues[19] demonstrated a 10-fold increase in prostaglandin E2 synthesis from labeled arachidonic acid in malignant human prostatic tissues. Shaw and colleagues[20] also showed increased prostaglandin E2 levels in the effusions of a fast-growing, metastasizing subline of the Dunning R-3327 rat prostatic adenocarcinoma when compared to a slow-growing derivative. Taken together, these findings are consistent with our observation of enhanced expression of sPLA2 immunoreactivity in high-grade PIN and prostatic adenocarcinoma.

sPLA2 has previously been localized using immunohistochemistry using human Paneth cells, chondrocytes, amniotic epithelial cells, and lacrimal gland cells.[21–23] Expression of different sPLA2 mRNA isoforms have been evaluated in various tissues.[24] Comparative expression of sPLA2 protein expression in other organs and their malignant counterparts has been published.[25–29] The distinctive staining pattern of group IIA sPLA2 between the benign and malignant prostatic glands suggests a utility of this antigen as a potentially useful diagnostic maker. Given the significant differences evident in the staining patterns, sPLA2 immunostaining might have an advantage over prostate-specific antigen and prostate-specific membrane antigen to differentiate between benign epithelium, high-grade PIN, and malignant prostatic cells. If present at all, benign epithelia show mainly focal sPLA2 staining. Even when positive, the staining intensity in benign epithelium is usually much weaker (one or two grades) than in adjacent malignant tissues. Because a minority (8.7% for high-grade PIN and 9.0% for adenocarcinoma) of the neoplastic cases were not immunoreactive, sPLA2 staining is not able to detect all of the prostatic cancers or high-grade PIN lesions. Further, a number of cases showed weak or moderate sPLA2 immunoreactivity in benign epithelium. Therefore, the utility of sPLA2 as a diagnostic marker to distinguish benign from neoplastic glands is still limited.

In summary, group IIA sPLA2 expression is elevated in the neoplastic human prostatic tissue raising the possibility that dysregulation of this enzyme may play a role in prostatic carcinogenesis. Our findings may have implications for target validation and development of therapeutic strategies modulating phospholipid metabolic pathways in prostatic neoplasia.

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The Hallmarks of Cancer

Review

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After a quarter century of rapid advances, cancer research has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome. The foundation has been set in the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function; both classes of cancer genes have been identified through their alteration in human and animal cancer cells and by their elicitation of cancer phenotypes in experimental models (Bishop and Weinberg, 1996).

Some would argue that the search for the origin and treatment of this disease will continue over the next quarter century in much the same manner as it has in the recent past, by adding further layers of complexity to a scientific literature that is already complex almost beyond measure. But we anticipate otherwise: those researching the cancer problem will be practicing a dramatically different type of science than we have experienced over the past 25 years. Surely much of this change will be apparent at the technical level. But ultimately, the more fundamental change will be conceptual.

We foresee cancer research developing into a logical science, where the complexities of the disease, described in the laboratory and clinic, will become understandable in terms of a small number of underlying principles. Some of these principles are even now in the midst of being codified. We discuss one set of them in the present essay: rules that govern the transformation of normal human cells into malignant cancers. We suggest that research over the past decades has revealed a small number of molecular, biochemical, and cellular traits—acquired capabilities—shared by most and perhaps all types of human cancer. Our faith in such simplification derives directly from the teachings of cell biology that virtually all mammalian cells carry a similar molecular machinery regulating their proliferation, differentiation, and death.

Several lines of evidence indicate that tumorigenesis in humans is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives. Many types of cancers are diagnosed in the human population with an age-dependent incidence implicating four to seven rate-limiting, stochastic events (Renan, 1993). Pathological analyses of a number of organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normalcy via a series of premalignant states into invasive cancers (Foulds, 1954).

These observations have been rendered more concrete by a large body of work indicating that the genomes of tumor cells are invariably altered at multiple sites, having suffered disruption through lesions as subtle as point mutations and as obvious as changes in chromosome complement (e.g., Kinzler and Vogelstein, 1996). Transformation of cultured cells is itself a multistep process: rodent cells require at least two introduced genetic changes before they acquire tumorigenic competence, while their human counterparts are more difficult to transform (Hahn et al., 1999). Transgenic models of tumorigenesis have repeatedly supported the conclusion that tumorigenesis in mice involves multiple rate-limiting steps (Bergers et al., 1998; see *Oncogene*, 1999, R. DePinho and T. E. Jacks, volume 18[38], pp. 5248–5362). Taken together, observations of human cancers and animal models argue that tumor development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Foulds, 1954; Nowell, 1976).

An Enumeration of the Traits

The barriers to development of cancer are embodied in a teleology: cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs. This complexity provokes a number of questions. How many distinct regulatory circuits within each type of target cell must be disrupted in order for such a cell to become cancerous? Does the same set of cellular regulatory circuits suffer disruption in the cells of the disparate neoplasms arising in the human body? Which of these circuits operate on a cell-autonomous basis, and which are coupled to the signals that cells receive from their surrounding microenvironment within a tissue? Can the large and diverse collection of cancer-associated genes be tied to the operations of a small group of regulatory circuits?

We suggest that the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth (Figure 1): self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes—novel capabilities acquired during tumor development—represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues. We propose that these six capabilities are shared in common by most and perhaps all types of human tumors. This multiplicity of defenses may explain why cancer is relatively rare during an average human lifetime.

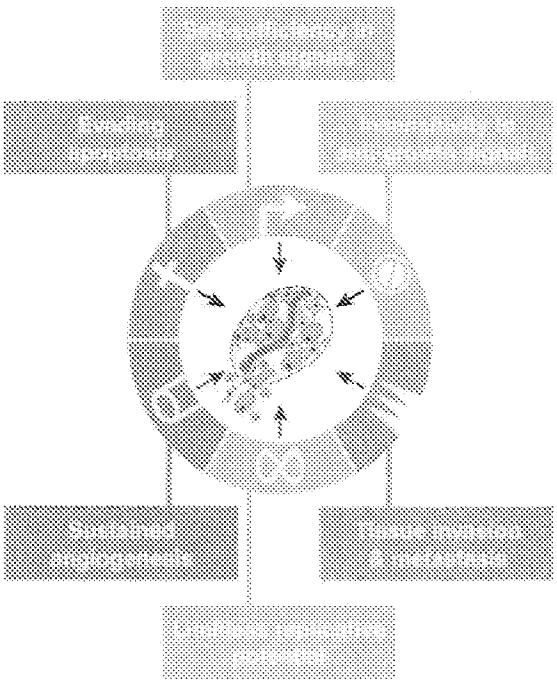

Figure 1. Acquired Capabilities of Cancer

We suggest that most if not all cancers have acquired the same set of functional capabilities during their development, albeit through various mechanistic strategies.

We describe each capability in turn below, illustrate with a few examples its functional importance, and indicate strategies by which it is acquired in human cancers.

Acquired Capability: Self-Sufficiency in Growth Signals

Normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. To our knowledge, no type of normal cell can proliferate in the absence of such stimulatory signals. Many of the oncogenes in the cancer catalog act by mimicking normal growth signaling in one way or another.

Dependence on growth signaling is apparent when propagating normal cells in culture, which typically proliferate only when supplied with appropriate diffusible mitogenic factors and a proper substratum for their integrins. Such behavior contrasts strongly with that of tumor cells, which invariably show a greatly reduced dependence on exogenous growth stimulation. The conclusion is that tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their normal tissue microenvironment. This liberation from dependence on exogenously derived signals disrupts a critically important homeostatic mechanism that normally operates to ensure a proper behavior of the various cell types within a tissue.

Acquired GS autonomy was the first of the six capabilities to be clearly defined by cancer researchers, in large part because of the prevalence of dominant oncogenes that have been found to modulate it. Three common molecular strategies for achieving autonomy are evident, involving alteration of extracellular growth signals, of transcellular transducers of those signals, or of intracellular circuits that translate those signals into action. While most soluble mitogenic growth factors (GFs) are made by one cell type in order to stimulate proliferation of another—the process of heterotypic signaling—many cancer cells acquire the ability to synthesize GFs to which they are responsive, creating a positive feedback signaling loop often termed autocrine stimulation (Fedi et al., 1997). Clearly, the manufacture of a GF by a cancer cell obviates dependence on GFs from other cells within the tissue. The production of PDGF (platelet-derived growth factor) and TGFα (tumor growth factor α) by glioblastomas and sarcomas, respectively, are two illustrative examples (Fedi et al., 1997).

The cell surface receptors that transduce growth-stimulatory signals into the cell interior are themselves targets of deregulation during tumor pathogenesis. GF receptors, often carrying tyrosine kinase activities in their cytoplasmic domains, are overexpressed in many cancers. Receptor overexpression may enable the cancer cell to become hyperresponsive to ambient levels of GF that normally would not trigger proliferation (Fedi et al., 1997). For example, the epidermal GF receptor (EGF-R/*erbB*) is upregulated in stomach, brain, and breast tumors, while the HER2/*neu* receptor is overexpressed in stomach and mammary carcinomas (Slamon et al., 1987; Yarden and Ullrich, 1988). Additionally, gross overexpression of GF receptors can elicit ligand-independent signaling (DiFiore et al., 1987). Ligand-independent signaling can also be achieved through structural alteration of receptors; for example, truncated versions of the EGF receptor lacking much of its cytoplasmic domain fire constitutively (Fedi et al., 1997).

Cancer cells can also switch the types of extracellular matrix receptors (integrins) they express, favoring ones that transmit progrowth signals (Lukashev and Werb, 1998; Giancotti and Ruoslahti, 1999). These bifunctional, heterodimeric cell surface receptors physically link cells to extracellular superstructures known as the extracellular matrix (ECM). Successful binding to specific moieties of the ECM enables the integrin receptors to transduce signals into the cytoplasm that influence cell behavior, ranging from quiescence in normal tissue to motility, resistance to apoptosis, and entrance into the active cell cycle. Conversely, the failure of integrins to forge these extracellular links can impair cell motility, induce apoptosis, or cause cell cycle arrest (Giancotti and Ruoslahti, 1999). Both ligand-activated GF receptors and progrowth integrins engaged to extracellular matrix components can activate the SOS-Ras-Raf-MAP kinase pathway (Aplin et al., 1998; Giancotti and Ruoslahti, 1999).

The most complex mechanisms of acquired GS autonomy derive from alterations in components of the downstream cytoplasmic circuitry that receives and processes the signals emitted by ligand-activated GF receptors and integrins. The SOS-Ras-Raf-MAPK cascade plays a central role here. In about 25% of human

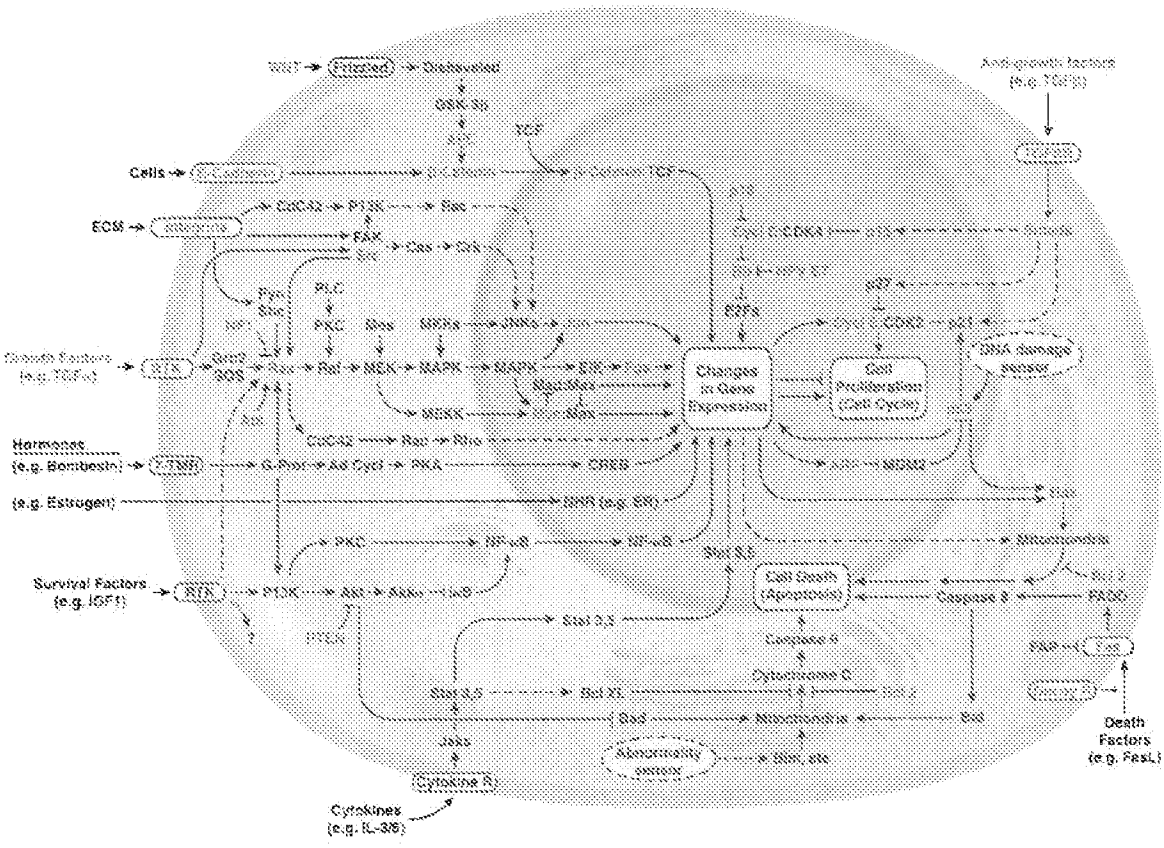

Figure 2. The Emergent Integrated Circuit of the Cell

Progress in dissecting signaling pathways has begun to lay out a circuitry that will likely mimic electronic integrated circuits in complexity and finesse, where transistors are replaced by proteins (e.g., kinases and phosphatases) and the electrons by phosphates and lipids, among others. In addition to the prototypical growth signaling circuit centered around Ras and coupled to a spectrum of extracellular cues, other component circuits transmit antigrowth and differentiation signals or mediate commands to live or die by apoptosis. As for the genetic reprogramming of this integrated circuit in cancer cells, some of the genes known to be functionally altered are highlighted in red.

tumors, Ras proteins are present in structurally altered forms that enable them to release a flux of mitogenic signals into cells, without ongoing stimulation by their normal upstream regulators (Medema and Bos, 1993).

We suspect that growth signaling pathways suffer deregulation in all human tumors. Although this point is hard to prove rigorously at present, the clues are abundant (Hunter, 1997). For example, in the best studied of tumors—human colon carcinomas—about half of the tumors bear mutant *ras* oncogenes (Kinzler and Vogelstein, 1996). We suggest that the remaining colonic tumors carry defects in other components of the growth signaling pathways that phenocopy *ras* oncogene activation. The nature of these alternative, growth-stimulating mechanisms remains elusive.

Under intensive study for two decades, the wiring diagram of the growth signaling circuitry of the mammalian cell is coming into focus (Figure 2). New downstream effector pathways that radiate from the central SOS-Ras-Raf-MAP kinase mitogenic cascade are being discovered with some regularity (Hunter, 1997; Rommel and Hafen, 1998). This cascade is also linked via a variety of cross-talking connections with other pathways; these cross connections enable extracellular signals to elicit multiple cell biological effects. For example, the direct interaction of the Ras protein with the survival-promoting PI3 kinase enables growth signals to concurrently evoke survival signals within the cell (Downward, 1998).

While acquisition of growth signaling autonomy by cancer cells is conceptually satisfying, it is also too simplistic. We have traditionally explored tumor growth by focusing our experimental attentions on the genetically deranged cancer cells (Figure 3, left panel). It is, however, increasingly apparent that the growth deregulation within a tumor can only be explained once we understand the contributions of the ancillary cells present in a tumor—the apparently normal bystanders such as fibroblasts and endothelial cells—which must play key roles in driving tumor cell proliferation (Figure 3, right panel). Within normal tissue, cells are largely instructed to grow by their neighbors (paracrine signals) or via systemic (endocrine) signals. Cell-to-cell growth signaling is likely to operate in the vast majority of human tumors as well; virtually all are composed of several distinct cell types that appear to communicate via heterotypic signaling.

Heterotypic signaling between the diverse cell types within a tumor may ultimately prove to be as important

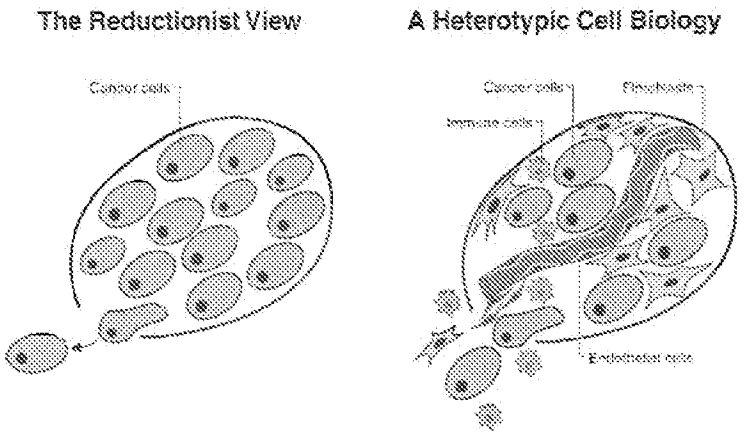

Figure 3. Tumors as Complex Tissues

The field of cancer research has largely been guided by a reductionist focus on cancer cells and the genes within them (left panel)—a focus that has produced an extraordinary body of knowledge. Looking forward in time, we believe that important new inroads will come from regarding tumors as complex tissues in which mutant cancer cells have conscripted and subverted normal cell types to serve as active collaborators in their neoplastic agenda (right panel). The interactions between the genetically altered malignant cells and these supporting coconspirators will prove critical to understanding cancer pathogenesis and to the development of novel, effective therapies.

in explaining tumor cell proliferation as the cancer cell-autonomous mechanisms enumerated above. For example, we suspect that many of the growth signals driving the proliferation of carcinoma cells originate from the stromal cell components of the tumor mass. While difficult to validate at present, such thinking recasts the logic of acquired GS autonomy: successful tumor cells are those that have acquired the ability to co-opt their normal neighbors by inducing them to release abundant fluxes of growth-stimulating signals (Skobe and Fusenig, 1998). Indeed, in some tumors, these cooperating cells may eventually depart from normalcy, coevolving with their malignant neighbors in order to sustain the growth of the latter (Kinzler and Vogelstein, 1996; Olumi et al., 1999). Further, inflammatory cells attracted to sites of neoplasia may promote (rather than eliminate) cancer cells (Cordon-Cardo and Prives, 1999; Coussens et al., 1999; Hudson et al., 1999), another example of normal cells conscripted to enhance tumor growth potential, another means to acquire necessary capabilities.

Acquired Capability: Insensitivity to Antigrowth Signals

Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. These growth-inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signaling circuits.

Antigrowth signals can block proliferation by two distinct mechanisms. Cells may be forced out of the active proliferative cycle into the quiescent (G_0) state from which they may reemerge on some future occasion when extracellular signals permit. Alternatively, cells may be induced to permanently relinquish their proliferative potential by being induced to enter into postmitotic states, usually associated with acquisition of specific differentiation-associated traits.

Incipient cancer cells must evade these antiproliferative signals if they are to prosper. Much of the circuitry that enables normal cells to respond to antigrowth signals is associated with the cell cycle clock, specifically the components governing the transit of the cell through the G1 phase of its growth cycle. Cells monitor their external environment during this period and, on the basis of sensed signals, decide whether to proliferate, to be quiescent, or to enter into a postmitotic state. At the molecular level, many and perhaps all antiproliferative signals are funneled through the retinoblastoma protein (pRb) and its two relatives, p107 and p130. When in a hypophosphorylated state, pRb blocks proliferation by sequestering and altering the function of E2F transcription factors that control the expression of banks of genes essential for progression from G1 into S phase (Weinberg, 1995).

Disruption of the pRb pathway liberates E2Fs and thus allows cell proliferation, rendering cells insensitive to antigrowth factors that normally operate along this pathway to block advance through the G1 phase of the cell cycle. The effects of the soluble signaling molecule TGFβ are the best documented, but we envision other antigrowth factors will be found to signal through this pathway as well. TGFβ acts in a number of ways, most still elusive, to prevent the phosphorylation that inactivates pRb; in this fashion, TGFβ blocks advance through G1. In some cell types, TGFβ suppresses expression of the *c-myc* gene, which regulates the G1 cell cycle machinery in still unknown ways (Moses et al., 1990). More directly, TGFβ causes synthesis of the $p15^{INK4B}$ and p21 proteins, which block the cyclin:CDK complexes responsible for pRb phosphorylation (Hannon and Beach, 1994; Datto et al., 1997).

The pRb signaling circuit, as governed by TGFβ and other extrinsic factors, can be disrupted in a variety of ways in different types of human tumors (Fynan and Reiss, 1993). Some lose TGFβ responsiveness through downregulation of their TGFβ receptors, while others display mutant, dysfunctional receptors (Fynan and Reiss, 1993; Markowitz et al., 1995). The cytoplasmic Smad4 protein, which transduces signals from ligand-activated TGFβ receptors to downstream targets, may be eliminated through mutation of its encoding gene (Schutte et al., 1996). The locus encoding $p15^{INK4B}$ may be deleted (Chin et al., 1998). Alternatively, the immediate downstream target of its actions, CDK4, may become unresponsive to the inhibitory actions of $p15^{INK4B}$ because of mutations that create amino acid substitutions

in its INK4A/B-interacting domain; the resulting cyclin D:CDK4 complexes are then given a free hand to inactivate pRb by hyperphosphorylation (Zuo et al., 1996). Finally, functional pRb, the end target of this pathway, may be lost through mutation of its gene. Alternatively, in certain DNA virus-induced tumors, notably cervical carcinomas, pRb function is eliminated through sequestration by viral oncoproteins, such as the E7 oncoprotein of human papillomavirus (Dyson et al., 1989). In addition, cancer cells can also turn off expression of integrins and other cell adhesion molecules that send antigrowth signals, favoring instead those that convey progrowth signals; these adherence-based antigrowth signals likely impinge on the pRb circuit as well. The bottom line is that the antigrowth circuit converging onto Rb and the cell division cycle is, one way or another, disrupted in a majority of human cancers, defining the concept and a purpose of tumor suppressor loss in cancer.

Cell proliferation depends on more than an avoidance of cytostatic antigrowth signals. Our tissues also constrain cell multiplication by instructing cells to enter irreversibly into postmitotic, differentiated states, using diverse mechanisms that are incompletely understood; it is apparent that tumor cells use various strategies to avoid this terminal differentiation. One strategy for avoiding differentiation directly involves the *c-myc* oncogene, which encodes a transcription factor. During normal development, the growth-stimulating action of Myc, in association with another factor, Max, can be supplanted by alternative complexes of Max with a group of Mad transcription factors; the Mad–Max complexes elicit differentiation-inducing signals (Foley and Eisenman, 1999). However, overexpression of the c-Myc oncoprotein, as is seen in many tumors, can reverse this process, shifting the balance back to favor Myc–Max complexes, thereby impairing differentiation and promoting growth. During human colon carcinogenesis, inactivation of the APC/β-catenin pathway serves to block the egress of enterocytes in the colonic crypts into a differentiated, postmitotic state (Kinzler and Vogelstein, 1996). Analogously, during the generation of avian erythroblastosis, the *erbA* oncogene acts to prevent irreversible erythrocyte differentiation (Kahn et al., 1986).

While the components and interconnections between the various antigrowth and differentiation-inducing signals and the core cell cycle machinery are still being delineated, the existence of an antigrowth signaling circuitry is clear (Figure 2), as is the necessity for its circumvention by developing cancers.

Acquired Capability: Evading Apoptosis

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death—apoptosis—represents a major source of this attrition. The evidence is mounting, principally from studies in mouse models and cultured cells, as well as from descriptive analyses of biopsied stages in human carcinogenesis, that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer.

Observations accumulated over the past decade indicate that the apoptotic program is present in latent form in virtually all cell types throughout the body. Once triggered by a variety of physiologic signals, this program unfolds in a precisely choreographed series of steps. Cellular membranes are disrupted, the cytoplasmic and nuclear skeletons are broken down, the cytosol is extruded, the chromosomes are degraded, and the nucleus is fragmented, all in a span of 30–120 min. In the end, the shriveled cell corpse is engulfed by nearby cells in a tissue and disappears, typically within 24 hr (Wyllie et al., 1980).

The apoptotic machinery can be broadly divided into two classes of components—sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. These signals regulate the second class of components, which function as effectors of apoptotic death. The sentinels include cell surface receptors that bind survival or death factors. Examples of these ligand/receptor pairs include survival signals conveyed by IGF-1/IGF-2 through their receptor, IGF-1R, and by IL-3 and its cognate receptor, IL-3R (Lotem and Sachs, 1996; Butt et al., 1999). Death signals are conveyed by the FAS ligand binding the FAS receptor and by TNFα binding TNF-R1 (Ashkenazi and Dixit, 1999). Intracellular sensors monitor the cell's well-being and activate the death pathway in response to detecting abnormalities, including DNA damage, signaling imbalance provoked by oncogene action, survival factor insufficiency, or hypoxia (Evan and Littlewood, 1998). Further, the life of most cells is in part maintained by cell–matrix and cell–cell adherence-based survival signals whose abrogation elicits apoptosis (Ishizaki et al., 1995; Giancotti and Ruoslahti, 1999). Both soluble and immobilized apoptotic regulatory signals likely reflect the needs of tissues to maintain their constituent cells in appropriate architectural configurations.

Many of the signals that elicit apoptosis converge on the mitochondria, which respond to proapoptotic signals by releasing cytochrome C, a potent catalyst of apoptosis (Green and Reed, 1998). Members of the Bcl-2 family of proteins, whose members have either proapoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) function, act in part by governing mitochondrial death signaling through cytochrome C release. The p53 tumor suppressor protein can elicit apoptosis by upregulating expression of proapoptotic Bax in response to sensing DNA damage; Bax in turn stimulates mitochondria to release cytochrome C.

The ultimate effectors of apoptosis include an array of intracellular proteases termed caspases (Thornberry and Lazebnik, 1998). Two "gatekeeper" caspases, −8 and −9, are activated by death receptors such as FAS or by the cytochrome C released from mitochondria, respectively. These proximal caspases trigger the activation of a dozen or more effector caspases that execute the death program, through selective destruction of subcellular structures and organelles, and of the genome.

The possibility that apoptosis serves as a barrier to cancer was first raised in 1972, when Kerr, Wyllie, and Currie described massive apoptosis in the cells populating rapidly growing, hormone-dependent tumors following hormone withdrawal (Kerr et al., 1972). The discovery

of the *bcl-2* oncogene by its upregulation via chromosomal translocation in follicular lymphoma (reviewed in Korsmeyer, 1992) and its recognition as having antiapoptotic activity (Vaux et al., 1988) opened up the investigation of apoptosis in cancer at the molecular level. When coexpressed with a *myc* oncogene in transgenic mice, the *bcl-2* gene was able to promote formation of B cell lymphomas by enhancing lymphocyte survival, not by further stimulating their *myc*-induced proliferation (Strasser et al., 1990); further, 50% of the infrequent lymphomas arising in *bcl-2* single transgenic transgenic mice had somatic translocations activating *c-myc*, confirming a selective pressure during lymphomagenesis to upregulate both Bcl-2 and c-Myc (McDonnell and Korsmeyer, 1991).

Further insight into the *myc-bcl-2* interaction emerged later from studying the effects of a *myc* oncogene on fibroblasts cultured in low serum. Widespread apoptosis was induced in *myc*-expressing cells lacking serum; the consequent apoptosis could be abrogated by exogenous survival factors (e.g., IGF-1), by forced overexpression of Bcl-2 or the related Bcl-XL protein, or by disruption of the FAS death signaling circuit (Hueber et al., 1997). Collectively, the data indicate that a cell's apoptotic program can be triggered by an overexpressed oncogene. Indeed, elimination of cells bearing activated oncogenes by apoptosis may represent the primary means by which such mutant cells are continually culled from the body's tissues.

Other examples strengthen the consensus that apoptosis is a major barrier to cancer that must be circumvented. Thus, in transgenic mice where the pRb tumor suppressor was functionally inactivated in the choroid plexus, slowly growing microscopic tumors arose, exhibiting high apoptotic rates; the additional inactivation of the p53 tumor suppressor protein, a component of the apoptotic signaling circuitry, led to rapidly growing tumors containing low numbers of apoptotic cells (Symonds et al., 1994). The role of extracellular survival factors is illustrated by disease progression in transgenic mice prone to pancreatic islet tumors. If *IGF-2* gene expression, which is activated in this tumorigenesis pathway, was abrogated using gene knockout mice, tumor growth and progression were impaired, as evidenced by the appearance of comparatively small, benign tumors showing high rates of apoptosis (Christofori et al., 1994). In these cells, the absence of IGF-2 did not affect cell proliferation rates, clearly identifying it as an antiapoptotic survival factor. Collectively, these observations argue that altering components of the apoptotic machinery can dramatically affect the dynamics of tumor progression, providing a rationale for the inactivation of this machinery during tumor development.

Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. Surely, the most commonly occurring loss of a proapoptotic regulator through mutation involves the *p53* tumor suppressor gene. The resulting functional inactivation of its product, the p53 protein, is seen in greater than 50% of human cancers and results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Harris, 1996). Signals evoked by other abnormalities, including hypoxia and oncogene hyperexpression, are also funneled in part via p53 to the apoptotic machinery; these too are impaired at eliciting apoptosis when p53 function is lost (Levine, 1997). Additionally, the PI3 kinase–AKT/PKB pathway, which transmits antiapoptotic survival signals, is likely involved in mitigating apoptosis in a substantial fraction of human tumors. This survival signaling circuit can be activated by extracellular factors such as IGF-1/2 or IL-3 (Evan and Littlewood, 1998), by intracellular signals emanating from Ras (Downward, 1998), or by loss of the pTEN tumor suppressor, a phospholipid phosphatase that normally attenuates the AKT survival signal (Cantley and Neel, 1999). Recently, a mechanism for abrogating the FAS death signal has been revealed in a high fraction of lung and colon carcinoma cell lines: a nonsignaling decoy receptor for FAS ligand is upregulated, titrating the death-inducing signal away from the FAS death receptor (Pitti et al., 1998). We expect that virtually all cancer cells harbor alterations that enable evasion of apoptosis.

It is now possible to lay out a provisional apoptotic signaling circuitry (Figure 2); while incomplete, it is evident that most regulatory and effector components are present in redundant form. This redundancy holds important implications for the development of novel types of antitumor therapy, since tumor cells that have lost proapoptotic components are likely to retain other similar ones. We anticipate that new technologies will be able to display the apoptotic pathways still operative in specific types of cancer cells and that new drugs will enable cross-talk between the still intact components of parallel apoptotic signaling pathways in tumor cells, resulting in restoration of the apoptotic defense mechanism, with substantial therapeutic benefit.

Acquired Capability: Limitless Replicative Potential

Three acquired capabilities—growth signal autonomy, insensitivity to antigrowth signals, and resistance to apoptosis—all lead to an uncoupling of a cell's growth program from signals in its environment. In principle, the resulting deregulated proliferation program should suffice to enable the generation of the vast cell populations that constitute macroscopic tumors. However, research performed over the past 30 years indicates that this acquired disruption of cell-to-cell signaling, on its own, does not ensure expansive tumor growth. Many and perhaps all types of mammalian cells carry an intrinsic, cell-autonomous program that limits their multiplication. This program appears to operate independently of the cell-to-cell signaling pathways described above. It too must be disrupted in order for a clone of cells to expand to a size that constitutes a macroscopic, life-threatening tumor.

The early work of Hayflick demonstrated that cells in culture have a finite replicative potential (reviewed in Hayflick, 1997). Once such cell populations have progressed through a certain number of doublings, they stop growing—a process termed senescence. The senescence of cultured human fibroblasts can be circumvented by disabling their pRb and p53 tumor suppressor proteins, enabling these cells to continue multiplying for additional generations until they enter into a second

state termed crisis. The crisis state is characterized by massive cell death, karyotypic disarray associated with end-to-end fusion of chromosomes, and the occasional emergence of a variant (1 in 10^7) cell that has acquired the ability to multiply without limit, the trait termed immortalization (Wright et al., 1989).

Provocatively, most types of tumor cells that are propagated in culture appear to be immortalized, suggesting that limitless replicative potential is a phenotype that was acquired in vivo during tumor progression and was essential for the development of their malignant growth state (Hayflick, 1997). This result suggests that at some point during the course of multistep tumor progression, evolving premalignant cell populations exhaust their endowment of allowed doublings and can only complete their tumorigenic agenda by breaching the mortality barrier and acquiring unlimited replicative potential.

Observations of cultured cells indicate that various normal human cell types have the capacity for 60–70 doublings. Taken at face value, these numbers make little sense when attempting to invoke cell mortality as an impediment to cancer formation: 60–70 doublings should enable clones of tumor cells to expand to numbers that vastly exceed the number of cells in the human body. If clues from evaluation of proliferation and apoptotic rates in certain human tumors (Wyllie et al., 1980) and transgenic mouse models (Symonds et al., 1994; Shibata et al., 1996; Bergers et al., 1998) prove generalizable, the paradox can be resolved: evolving premalignant and malignant cell populations evidence chronic, widespread apoptosis and consequently suffer considerable cell attrition concomitant with cell accumulation. Thus, the number of cells in a tumor greatly underrepresents the cell generations required to produce it, raising the generational limit of normal somatic cells as a barrier to cancer.

The counting device for cell generations has been discovered over the past decade: the ends of chromosomes, telomeres, which are composed of several thousand repeats of a short 6 bp sequence element. Replicative generations are counted by the 50–100 bp loss of telomeric DNA from the ends of every chromosome during each cell cycle. This progressive shortening has been attributed to the inability of DNA polymerases to completely replicate the 3′ ends of chromosomal DNA during each S phase. The progressive erosion of telomeres through successive cycles of replication eventually causes them to lose their ability to protect the ends of chromosomal DNA. The unprotected chromosomal ends participate in end-to-end chromosomal fusions, yielding the karyotypic disarray associated with crisis and resulting, almost inevitably, in the death of the affected cell (Counter et al., 1992).

Telomere maintenance is evident in virtually all types of malignant cells (Shay and Bacchetti, 1997); 85%–90% of them succeed in doing so by upregulating expression of the telomerase enzyme, which adds hexanucleotide repeats onto the ends of telomeric DNA (Bryan and Cech, 1999), while the remainder have invented a way of activating a mechanism, termed ALT, which appears to maintain telomeres through recombination-based interchromosomal exchanges of sequence information (Bryan et al., 1995). By one or the other mechanism, telomeres are maintained at a length above a critical threshold, and this in turn permits unlimited multiplication of descendant cells. Both mechanisms seem to be strongly suppressed in most normal human cells in order to deny them unlimited replicative potential.

The role of telomerase in immortalizing cells can be demonstrated directly by ectopically expressing the enzyme in cells, where it can convey unlimited replicative potential onto a variety of normal early passage, presenescent cells in vitro (Bodnar et al., 1998; Vaziri and Benchimol, 1998). Further, late passage cells poised to enter crisis continue to proliferate without giving any evidence of crisis when supplied with this enzyme (Counter et al., 1998; Halvorsen et al., 1999; Zhu et al., 1999). Additional clues into the importance of telomere maintenance for cancer comes from analysis of mice lacking telomerase function. For example, mice carrying a homozygous knockout of the cell cycle inhibitor $p16^{INK4A}$ are tumor prone, particularly when exposed to carcinogens; the tumors that arise show comparatively elevated telomerase activity. When carcinogens were applied to $p16^{INK4A}$-null mice that also lacked telomerase, tumor incidence was reduced, concomitant with substantial telomere shortening and karyotypic disarray in those tumors that did appear (Greenberg et al., 1999).

While telomere maintenance is clearly a key component of the capability for unlimited replication, we remain uncertain about another one, the circumvention of cellular senescence. The phenomenon of senescence was originally observed as a delayed response of primary cells to extended propagation in vitro and has thus been associated with mechanisms of divisional counting (Hayflick, 1997). More recently, the senescent state has been observed to be inducible in certain cultured cells in response to high level expression of genes such as the activated *ras* oncogene (Serrano et al., 1997).

The above-cited observations might argue that senescence, much like apoptosis, reflects a protective mechanism that can be activated by shortened telomeres or conflicting growth signals that forces aberrant cells irreversibly into a G_0-like state, thereby rendering them incapable of further proliferation. If so, circumvention of senescence in vivo may indeed represent an essential step in tumor progression that is required for the subsequent approach to and breaching of the crisis barrier. But we consider an alternative model equally plausible: senescence could be an artifact of cell culture that does not reflect a phenotype of cells within living tissues and does not represent an impediment to tumor progression in vivo. Resolution of this quandary will be critical to completely understand the acquisition of limitless replicative potential.

Acquired Capability: Sustained Angiogenesis

The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 μm of a capillary blood vessel. During organogenesis, this closeness is ensured by coordinated growth of vessels and parenchyma. Once a tissue is formed, the growth of new blood vessels—the process of angiogenesis—is transitory and carefully regulated. Because of this dependence on nearby capillaries, it would seem plausible that proliferating cells within a tissue would have an

intrinsic ability to encourage blood vessel growth. But the evidence is otherwise. The cells within aberrant proliferative lesions initially lack angiogenic ability, curtailing their capability for expansion. In order to progress to a larger size, incipient neoplasias must develop angiogenic ability (Bouck et al., 1996; Hanahan and Folkman, 1996; Folkman, 1997).

Counterbalancing positive and negative signals encourage or block angiogenesis. One class of these signals is conveyed by soluble factors and their receptors, the latter displayed on the surface of endothelial cells; integrins and adhesion molecules mediating cell–matrix and cell–cell association also play critical roles. The angiogenesis-initiating signals are exemplified by vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2). Each binds to transmembrane tyrosine kinase receptors displayed by endothelial cells (Fedi et al., 1997; Veikkola and Alitalo, 1999). A prototypical angiogenesis inhibitor is thrombospondin-1, which binds to CD36, a transmembrane receptor on endothelial cells coupled to intracellular Src-like tyrosine kinases (Bull et al., 1994). There are currently more than two dozen angiogenic inducer factors known and a similar number of endogenous inhibitor proteins.

Integrin signaling also contributes to this regulatory balance. Quiescent vessels express one class of integrins, whereas sprouting capillaries express another. Interference with signaling from the latter class of integrins can inhibit angiogenesis (Varner and Cheresh, 1996; Giancotti and Ruoslahti, 1999), underscoring the important contribution of cell adhesion to the angiogenic program (Hynes and Wagner, 1996). Extracellular proteases are physically and functionally connected with pro-angiogenic integrins, and both help dictate the invasive capability of angiogenic endothelial cells (Stetler-Stevenson, 1999).

Experimental evidence for the importance of inducing and sustaining angiogenesis in tumors is both extensive and compelling (Bouck et al., 1996; Hanahan and Folkman, 1996; Folkman, 1997). The story begins almost 30 years ago with Folkman and colleagues, who used in vivo bioassays to demonstrate the necessity of angiogenesis for explosive growth of tumor explants (reviewed in Folkman, 1997). Molecular proof of principle came, for example, when anti-VEGF antibodies proved able to impair neovascularization and growth of subcutaneous tumors in mice (Kim et al., 1993), as did a dominant-interfering version of the VEGF receptor 2 (flk-1) (Millauer et al., 1994); both results have motivated the development of specific VEGF/VEGF-R inhibitors now in late stage clinical trials.

The essential role of angiogenesis is further supported by the ability of an increasing catalog of antiangiogenic substances to impair the growth of tumor cells inoculated subcutaneously in mice (Folkman, 1997). Tumors arising in cancer-prone transgenic mice are similarly susceptible to angiogenic inhibitors (Bergers et al., 1999).

The ability to induce and sustain angiogenesis seems to be acquired in a discrete step (or steps) during tumor development, via an "angiogenic switch" from vascular quiescence. When three transgenic mouse models were analyzed throughout multistep tumorigenesis, in each case angiogenesis was found to be activated in mid-stage lesions, prior to the appearance of full-blown tumors. Similarly, angiogenesis can be discerned in pre-malignant lesions of the human cervix, breast, and skin (melanocytes) (Hanahan and Folkman, 1996); we expect that induction of angiogenesis will prove to be an early to midstage event in many human cancers. These observations, taken together with the effects of angiogenesis inhibitors, indicate that neovascularization is a prerequisite to the rapid clonal expansion associated with the formation of macroscopic tumors.

Tumors appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors (Hanahan and Folkman, 1996). One common strategy for shifting the balance involves altered gene transcription. Many tumors evidence increased expression of VEGF and/or FGFs compared to their normal tissue counterparts. In others, expression of endogenous inhibitors such as thrombospondin-1 or β-interferon is downregulated. Moreover, both transitions may occur, and indeed be linked, in some tumors (Singh et al., 1995; Volpert et al., 1997).

The mechanisms underlying shifts in the balances between angiogenic regulators remain incompletely understood. In one well-documented example, the inhibitor thrombospondin-1 has been found to positively regulated by the p53 tumor suppressor protein in some cell types. Consequently, loss of p53 function, which occurs in most human tumors, can cause thrombospondin-1 levels to fall, liberating endothelial cells from its inhibitory effects (Dameron et al., 1994). The *VEGF* gene is also under complex transcriptional control. For example, activation of the *ras* oncogene or loss of the VHL tumor suppressor gene in certain cell types causes upregulation of VEGF expression (Rak et al., 1995; Maxwell et al., 1999).

Another dimension of regulation is emerging in the form of proteases, which can control the bioavailability of angiogenic activators and inhibitors. Thus, a variety of proteases can release bFGF stored in the ECM (Whitelock et al., 1996), whereas plasmin, a proangiogenic component of the clotting system, can cleave itself into an angiogenesis inhibitor form called angiostatin (Gately et al., 1997). The coordinated expression of pro- and antiangiogenic signaling molecules, and their modulation by proteolysis, appear to reflect the complex homeostatic regulation of normal tissue angiogenesis and of vascular integrity.

As is already apparent, tumor angiogenesis offers a uniquely attractive therapeutic target, indeed one that is shared in common by most and perhaps all types of human tumors. The next decade will produce a catalog of the angiogenic regulatory molecules expressed by different types of tumors, and in many cases, by their progenitor stages. Use of increasingly sophisticated mouse models will make it possible to assign specific roles to each of these regulators and to discern the molecular mechanisms that govern their production and activity. Already available evidence indicates that different types of tumor cells use distinct molecular strategies to activate the angiogenic switch. This raises the question of whether a single antiangiogenic therapeutic will suffice to treat all tumor types, or whether an ensemble of such therapeutics will need to be developed, each

responding to a distinct program of angiogenesis that has been developed by a specific class of human tumors.

Acquired Capability: Tissue Invasion and Metastasis

Sooner or later during the development of most types of human cancer, primary tumor masses spawn pioneer cells that move out, invade adjacent tissues, and thence travel to distant sites where they may succeed in founding new colonies. These distant settlements of tumor cells—metastases—are the cause of 90% of human cancer deaths (Sporn, 1996). The capability for invasion and metastasis enables cancer cells to escape the primary tumor mass and colonize new terrain in the body where, at least initially, nutrients and space are not limiting. The newly formed metastases arise as amalgams of cancer cells and normal supporting cells conscripted from the host tissue. Like the formation of the primary tumor mass, successful invasion and metastasis depend upon all of the other five acquired hallmark capabilities. But what additional cellular changes enable the acquisition of these final capabilities during tumorigenesis?

Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. At the mechanistic level, they are closely allied processes, which justifies their association with one another as one general capability of cancer cells. Both utilize similar operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases.

Several classes of proteins involved in the tethering of cells to their surroundings in a tissue are altered in cells possessing invasive or metastatic capabilities. The affected proteins include cell–cell adhesion molecules (CAMs)—notably members of the immunoglobulin and calcium-dependent cadherin families, both of which mediate cell-to-cell interactions—and integrins, which link cells to extracellular matrix substrates. Notably, all of these "adherence" interactions convey regulatory signals to the cell (Aplin et al., 1998). The most widely observed alteration in cell-to-environment interactions in cancer involves E-cadherin, a homotypic cell-to-cell interaction molecule ubiquitously expressed on epithelial cells. Coupling between adjacent cells by E-cadherin bridges results in the transmission of antigrowth and other signals via cytoplasmic contacts with β-catenin to intracellular signaling circuits that include the Lef/Tcf transcription factor (Christofori and Semb, 1999). E-cadherin function is apparently lost in a majority of epithelial cancers, by mechanisms that include mutational inactivation of the E-cadherin or β-catenin genes, transcriptional repression, or proteolysis of the extracellular cadherin domain (Christofori and Semb, 1999). Forced expression of E-cadherin in cultured cancer cells and in a transgenic mouse model of carcinogenesis impairs invasive and metastatic phenotypes, whereas interference with E-cadherin function enhances both capabilities (Christofori and Semb, 1999). Thus, E-cadherin serves as a widely acting suppressor of invasion and metastasis by epithelial cancers, and its functional elimination represents a key step in the acquisition of this capability.

Changes in expression of CAMs in the immunoglobulin superfamily also appear to play critical roles in the processes of invasion and metastasis (Johnson, 1991). The clearest case involves N-CAM, which undergoes a switch in expression from a highly adhesive isoform to poorly adhesive (or even repulsive) forms in Wilms' tumor, neuroblastoma, and small cell lung cancer (Johnson, 1991; Kaiser et al., 1996) and reduction in overall expression level in invasive pancreatic and colorectal cancers (Fogar et al., 1997). Experiments in transgenic mice support a functional role for the normal adhesive form of N-CAM in suppressing metastasis (Perl et al., 1999).

Changes in integrin expression are also evident in invasive and metastatic cells. Invading and metastasizing cancer cells experience changing tissue microenvironments during their journeys, which can present novel matrix components. Accordingly, successful colonization of these new sites (both local and distant) demands adaptation, which is achieved through shifts in the spectrum of integrin α or β subunits displayed by the migrating cells. These novel permutations result in different integrin subtypes (of which there are greater than 22) having distinct substrate preferences. Thus, carcinoma cells facilitate invasion by shifting their expression of integrins from those that favor the ECM present in normal epithelium to other integrins (e.g., α3β1 and αVβ3) that preferentially bind the degraded stromal components produced by extracellular proteases (Varner and Cheresh, 1996; Lukashev and Werb, 1998). Forced expression of integrin subunits in cultured cells can induce or inhibit invasive and metastatic behavior, consistent with a role of these receptors in acting as central determinants of these processes (Varner and Cheresh, 1996).

Attempts at explaining the cell biological effects of integrins in terms of a small number of mechanistic rules have been confounded by the large number of distinct integrin genes, by the even larger number of heterodimeric receptors resulting from combinatorial expression of various α and β receptor subunits, and by the increasing evidence of complex signals emitted by the cytoplasmic domains of these receptors (Aplin et al., 1998; Giancotti and Ruoslahti, 1999). Still, there is little doubt that these receptors play central roles in the capability for tissue invasion and metastasis.

The second general parameter of the invasive and metastatic capability involves extracellular proteases (Coussens and Werb, 1996; Chambers and Matrisian, 1997). Protease genes are upregulated, protease inhibitor genes are downregulated, and inactive zymogen forms of proteases are converted into active enzymes. Matrix-degrading proteases are characteristically associated with the cell surface, by synthesis with a transmembrane domain, binding to specific protease receptors, or association with integrins (Werb, 1997; Stetler-Stevenson, 1999). One imagines that docking of active proteases on the cell surface can facilitate invasion by cancer cells into nearby stroma, across blood vessel walls, and through normal epithelial cell layers. That notion notwithstanding, it is difficult to unambiguously ascribe the functions of particular proteases solely to this capability, given their evident roles in other hallmark capabilities, including angiogenesis (Stetler-Stevenson, 1999) and growth signaling (Werb, 1997;

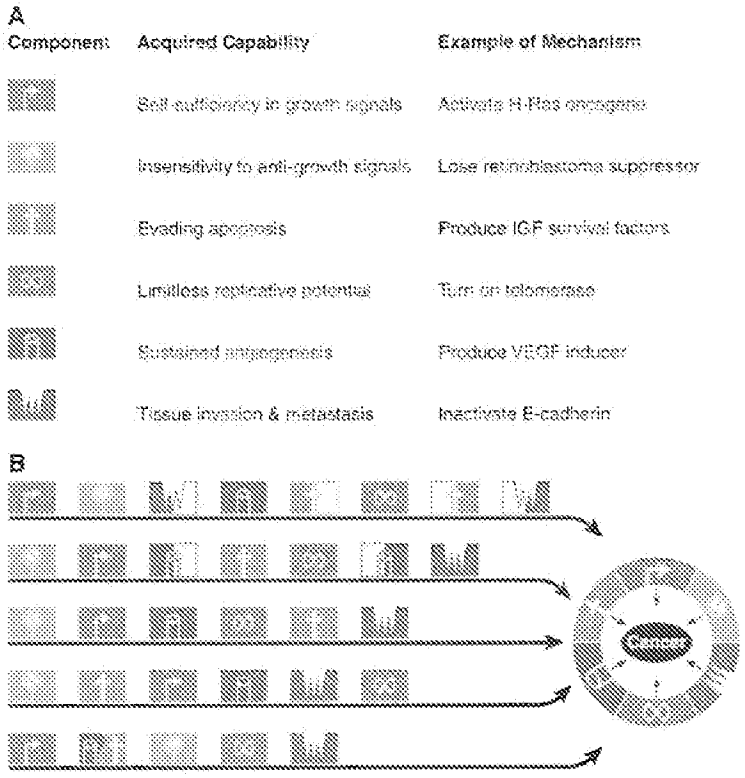

Figure 4. Parallel Pathways of Tumorigenesis

While we believe that virtually all cancers must acquire the same six hallmark capabilities (A), their means of doing so will vary significantly, both mechanistically (see text) and chronologically (B). Thus, the order in which these capabilities are acquired seems likely be quite variable across the spectrum of cancer types and subtypes. Moreover, in some tumors, a particular genetic lesion may confer several capabilities simultaneously, decreasing the number of distinct mutational steps required to complete tumorigenesis. Thus, loss of function of the p53 tumor suppressor can facilitate both angiogenesis and resistance to apoptosis (e.g., in the five-step pathway shown), as well as enabling the characteristic of genomic instability. In other tumors, a capability may only be acquired through the collaboration of two or more distinct genetic changes, thereby increasing the total number necessary for completion of tumor progression. Thus, in the eight-step pathway shown, invasion/metastasis and resistance to apoptosis are each acquired in two steps.

Bergers and Coussens, 2000), which in turn contribute directly or indirectly to the invasive/metastatic capability.

A further dimension of complexity derives from the multiple cell types involved in protease expression and display. In many types of carcinomas, matrix-degrading proteases are produced not by the epithelial cancer cells but rather by conscripted stromal and inflammatory cells (Werb, 1997); once released by these cells, they may be wielded by the carcinoma cells. For example, certain cancer cells induce urokinase (uPA) expression in cocultured stromal cells, which then binds to the urokinase receptor (uPAR) expressed on the cancer cells (Johnsen et al., 1998).

The activation of extracellular proteases and the altered binding specificities of cadherins, CAMs, and integrins are clearly central to the acquisition of invasiveness and metastatic ability. But the regulatory circuits and molecular mechanisms that govern these shifts remain elusive and, at present, seem to differ from one tissue environment to another. The acquired capability for invasion and metastasis represents the last great frontier for exploratory cancer research. We envision that evolving analytic techniques will soon make it possible to construct comprehensive profiles of the expression and functional activities of proteases, integrins, and CAMs in a wide variety of cancer types, both before and after they acquire invasive and metastatic abilities. The challenge will then be to apply the new molecular insights about tissue invasiveness and metastasis to the development of effective therapeutic strategies.

An Enabling Characteristic: Genome Instability

The acquisition of the enumerated six capabilities during the course of tumor progression creates a dilemma. The available evidence suggests that most are acquired, directly or indirectly, through changes in the genomes of cancer cells. But mutation of specific genes is an inefficient process, reflecting the unceasing, fastidious maintenance of genomic integrity by a complex array of DNA monitoring and repair enzymes. These genome maintenance teams strive to ensure that DNA sequence information remains pristine. Karyotypic order is guaranteed by yet other watchmen, manning so-called checkpoints, that operate at critical times in the cell's life, notably mitosis. Together, these systems ensure that mutations are rare events, indeed so rare that the multiple mutations known to be present in tumor cell genomes are highly unlikely to occur within a human life span.

Yet cancers do appear at substantial frequency in the human population, causing some to argue that the genomes of tumor cells must acquire increased mutability in order for the process of tumor progression to reach completion in several decades time (Loeb, 1991). Malfunction of specific components of these genomic "caretaker" systems has been invoked to explain this increased mutability (Lengauer et al., 1998). The most prominent member of these systems is the p53 tumor suppressor protein, which, in response to DNA damage, elicits either cell cycle arrest to allow DNA repair to take place or apoptosis if the damage is excessive. Indeed, it is now clear that the functioning of the p53 DNA damage signaling pathway is lost in most, if not all, human cancers (Levine, 1997). Moreover, a growing number of other genes involved in sensing and repairing DNA damage, or in assuring correct chromosomal segregation during mitosis, is found to be lost in different cancers, labeling these caretakers as tumor suppressors (Lengauer et al., 1998). Their loss of function is envisioned

to allow genome instability and variability and the generation of consequently mutant cells with selective advantages. Interestingly, recent evidence suggests that apoptosis may also be a vehicle of genomic instability, in that DNA within apoptotic cell bodies can be incorporated into neighboring cells following phagocytosis (Holmgren et al., 1999), in principle genetically diversifying any of the constituent cell types of a tumor. We place this acquired characteristic of genomic instability apart from the six acquired capabilities associated with tumor cell phenotype and tumor physiology: it represents the means that enables evolving populations of premalignant cells to reach these six biological endpoints.

Alternative Pathways to Cancer

The paths that cells take on their way to becoming malignant are highly variable. Within a given cancer type, mutation of particular target genes such as *ras* or *p53* may be found in only a subset of otherwise histologically identical tumors. Further, mutations in certain oncogenes and tumor suppressor genes can occur early in some tumor progression pathways and late in others. As a consequence, the acquisition of biological capabilities such as resistance to apoptosis, sustained angiogenesis, and unlimited replicative potential can appear at different times during these various progressions. Accordingly, the particular sequence in which capabilities are acquired can vary widely, both among tumors of the same type and certainly between tumors of different types (Figure 4). Furthermore, in certain tumors, a specific genetic event may, on its own, contribute only partially to the acquisition of a single capability, while in others, this event may aid in the simultaneous acquisition of several distinct capabilities. Nonetheless, we believe that independent of how the steps in these genetic pathways are arranged, the biological endpoints that are ultimately reached—the hallmark capabilities of cancer—will prove to be shared in common by all types of tumors.

Synthesis

Cancer cells propagated in culture and dissected into their molecular components have yielded much of the wealth of information that we currently possess about the molecular processes underlying cancer development. Yet by simplifying the nature of cancer—portraying it as a cell-autonomous process intrinsic to the cancer cell—these experimental models have turned their back on a central biological reality of tumor formation in vivo: cancer development depends upon changes in the heterotypic interactions between incipient tumor cells and their normal neighbors. Moreover, once formed, virtually all types of human tumors, including their metastatic outgrowths, continue to harbor complex mixtures of several cell types that collaborate to create malignant growth (Figure 3). This reconceptualization of cancer cell biology has begun to drive profound changes in how we study this disease experimentally. Continuing elucidation of cancer pathogenesis will depend increasingly upon heterotypic organ culture systems in vitro and evermore refined mouse models in vivo. Looking ahead into the future, these systems will help us chart comprehensive maps of growth signaling networks in cancer, an endeavor that will depend on defining all of the signals exchanged between the various cell types existing symbiotically within a tumor mass and knowing their effects on the integrated circuits of each of those cell types.

Our ability to analyze individual human cancers at the genetic and biochemical levels will also undergo a dramatic change. At present, description of a recently diagnosed tumor in terms of its underlying genetic lesions remains a distant prospect. Nonetheless, we look ahead 10 or 20 years to the time when the diagnosis of all the somatically acquired lesions present in a tumor cell genome will become a routine procedure. By then, genome-wide gene expression profiles of tumor cells will also be routine. With all this information in hand, it will become possible to test definitively our proposition that the development of all types of human tumor cells is governed by a common set of rules such as those implied by the six acquired capabilities enumerated here.

We anticipate far deeper insight into the roles played by inherited alleles in cancer susceptibility and pathogenesis. At present, our understanding of the interplay at the cellular level between inherited cancer modifier genes with oncogenes and tumor suppressor genes that are altered somatically is rudimentary; modifiers can in principle act in any of the constituent cell types of a tumor, or elsewhere in the body, whereas the classical cancer genes largely act in the cancer cells themselves. These gaps will be bridged in part by new informatics technologies, enabling us to process and interpret the inundation of genetic information that will soon flow from automated sequencing instruments. New technologies will also aid us in rationalizing the complex constellations of interacting alleles in terms of a systematics of cancer formation of the type that we propose here.

The metaphors used to conceptualize cancer cell function will also shift dramatically. For decades now, we have been able to predict with precision the behavior of an electronic integrated circuit in terms of its constituent parts—its interconnecting components, each responsible for acquiring, processing, and emitting signals according to a precisely defined set of rules. Two decades from now, having fully charted the wiring diagrams of every cellular signaling pathway, it will be possible to lay out the complete "integrated circuit of the cell" upon its current outline (Figure 2). We will then be able to apply the tools of mathematical modeling to explain how specific genetic lesions serve to reprogram this integrated circuit in each of the constituent cell types so as to manifest cancer.

With holistic clarity of mechanism, cancer prognosis and treatment will become a rational science, unrecognizable by current practitioners. It will be possible to understand with precision how and why treatment regimens and specific antitumor drugs succeed or fail. We envision anticancer drugs targeted to each of the hallmark capabilities of cancer; some, used in appropriate combinations and in concert with sophisticated technologies to detect and identify all stages of disease progression, will be able to prevent incipient cancers from developing, while others will cure preexisting cancers, elusive goals at present. One day, we imagine that cancer biology and treatment—at present, a patchwork quilt of cell biology, genetics, histopathology, biochemistry,

immunology, and pharmacology—will become a science with a conceptual structure and logical coherence that rivals that of chemistry or physics.

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RAPID COMMUNICATION

Role of Programmed (Apoptotic) Cell Death During the Progression and Therapy for Prostate Cancer

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ABSTRACT: Cells possess within their epigenetic repertoire the ability to undergo an active process of cellular suicide termed *programmed* (or apoptotic) *cell death*. This programmed cell death process involves an epigenetic reprogramming of the cell that results in an energy-dependent cascade of biochemical and morphologic changes (also termed apoptosis) within the cell, resulting in its death and elimination. Although the final steps (i.e., DNA and cellular fragmentation) are common to cells undergoing programmed cell death, the activation of this death process is initiated either by sufficient injury to the cell induced by various exogenous damaging agents (e.g., radiation, chemicals, viruses) or by changes in the levels of a series of endogenous signals (e.g., hormones and growth/survival factors). Within the prostate, androgens are capable of both stimulating proliferation as well as inhibiting the rate of the glandular epithelial cell death. Androgen withdrawal triggers the programmed cell death pathway in both normal prostate glandular epithelia and androgen-dependent prostate cancer cells. Androgen-independent prostate cancer cells do not initiate the programmed cell death pathway upon androgen ablation; however, they do retain the cellular machinery necessary to activate the programmed cell death cascade when sufficiently damaged by exogenous agents. In the normal prostate epithelium, cell proliferation is balanced by an equal rate of programmed cell death, such that neither involution nor overgrowth normal occurs. In prostatic cancer, however, this balance is lost, such that there is greater proliferation than death producing continuous net growth. Thus, an imbalance in programmed cell death must occur during prostatic cancer progression. The goal of effective therapy for prostatic cancer, therefore, is to correct this imbalance. Unfortunately, this has not been achieved and metastatic prostatic cancer is still a lethal disease for which no curative therapy is currently available. In order to develop such effective therapy, an understanding of the programmed death pathway, and what controls it, is critical. Thus, a review of the present state of knowledge concerning programmed cell death of normal and malignant prostatic cells will be presented.

KEY WORDS: **apoptosis, programmed cell death, prostatic cancer**

INTRODUCTION

The study of programmed cell death/apoptosis in both the normal prostate gland and in prostate cancer has become a major area of prostate research. The prostate gland affords a unique opportunity to study programmed cell death during the normal process of glandular self-renewal. At the same time, the prostate represents an unparalleled system for studying the mechanisms of programmed cell death in neoplasia, both in terms of response to an initially effective therapy, androgen ablation, and in subsequent resistance to programmed cell death with progression to

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an androgen-independent state. Currently, nearly all men with metastatic prostate cancer treated with surgical or medical castration have an initial beneficial response to androgen withdrawal. While this initial response has substantial palliative value, almost all treated patients relapse to an androgen-insensitive state. Unfortunately, once prostate cancer progresses to become androgen independent, it is uniformly fatal because no effective systemic therapy currently exists. Annually, an estimated 41,000 American men will die from prostate cancer [1]. This number represents 15% of all cancer deaths for men, making prostate cancer the second leading cause of cancer deaths in males [1]. Even more startling is the fact that approximately one out of every three newly diagnosed cancers in men will be due to prostate cancer [1]. Therefore, understanding the mechanisms of programmed cell death could prove critical to developing new, effective therapies for prostate cancer.

Unlike the detailed framework that is rapidly becoming defined in both molecular and cell biological terms for cell proliferation, an understanding of what initiates cell death and what the cellular mechanics are for this process is just beginning. Cell death can involve processes that are equal in complexity and regulation to those involved in cell proliferation. This knowledge has been appreciated for a number of years by developmental biologists. This group of scientists coined the term programmed cell death to distinguish the active, orderly, and cell-type-specific death observed in developing organisms from necrotic cell death. Necrotic death is a response to pathologic changes initiated outside of the cell and can be elicited by any of a large series of factors that result in a change in the plasma membrane permeability. This increased plasma membrane permeability results in cellular edema and in the eventual osmotic lysis of the cell. In necrotic cell death, the cell has a passive role in initiating the process of death. On the contrary, in programmed cell death, a cell undergoes an energy-dependent process of cellular suicide initiated by specific signals in an otherwise normal microenvironment. In programmed cell death the cell is an active participant in its own demise [2,3].

Programmed cell death is a widespread phenomenon occuring normally at different stages of morphogenesis, growth and development of metazoans, and in normal turnover in adult tissue [4]. Under these physiologic conditions, programmed cell death is initiated in specific cell types by both endogenous tissue-specific agents (generally hormones) and exogenous cell-damaging treatments (e.g., radiation, chemicals, and viruses). Endogenous activation of programmed cell death can occur due to either the positive presence of a tissue-specific inducer such as the induction of death in immature thymocytes by glucocorticoids [5] or to the negative lack of a tissue-specific repressor such as induction of death of prostatic glandular cells by androgen ablation [6]. Once initiated, programmed cell death leads to a cascade of biochemical and morphological events that result in irreversible degradation of the genomic DNA into discontinuous nucleosomal repeat ladders with subsequent fragmentation of the cell. The morphologic pathway for programmed cell death is rather stereotypical and has been given the name apoptosis to distinguish this process form necrotic cell death [2,3]. DNA is also degraded during necrotic cell death; however, this is a late event in necrotic cells whose plasma and internal membranes have already lysed. In necrotic death, DNA is degraded into a continuous spectrum of sizes as a result of the simultaneous action of lysosomal proteases and nucleases released in dead cells [5].

Apoptosis was originally defined by Kerr et al. [2] as the orderly and characteristic sequence of structural changes resulting in the programmed death of the cell. Morphologically, apoptosis is characterized by a temporal sequence of events consisting of chromatin aggregation, nuclear and cytoplasmic condensation, and eventual fragmentation of the dying cell into a cluster of membrane-bound segments (apoptotic bodies) that often contain morphologically intact organelles. These apoptotic bodies are rapidly recognized, phagocytized, and digested by either macrophages or adjacent epithelial cells. In programmed cell death, the cell also progresses through an orderly series of biochemical and molecular changes, similar to the sequential changes involved in progression through the proliferative cell cycle (Fig. 1). The hallmark of the programmed cell death process is the fragmentation of genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma and internal membrane permeability [4–7]. This period of DNA fragmentation (the F phase) (Fig. 1) can be used to divide the temporal series of events involved in programmed cell death, much as the period of DNA synthesis (the S phase) is used to divide the proliferative cell cycle. The overall cell cycle controlling cell number is thus composed of a multicompartment system in which the cell has at least three possible options (Fig. 1). The cell can be (1) metabolically active but not undergoing either proliferation or death (G_0 cell); (2) undergoing cell proliferation (G_0 to mitosis); or (3) undergoing cell death by either the programmed pathway (G_0-D1-F-D2 apoptotic cellular fragmentation) or the nonprogrammed (necrotic) pathway [8]. The endogenous systemic and local growth factor signals that regulate the progression within this cell cycle are cell type specific and are

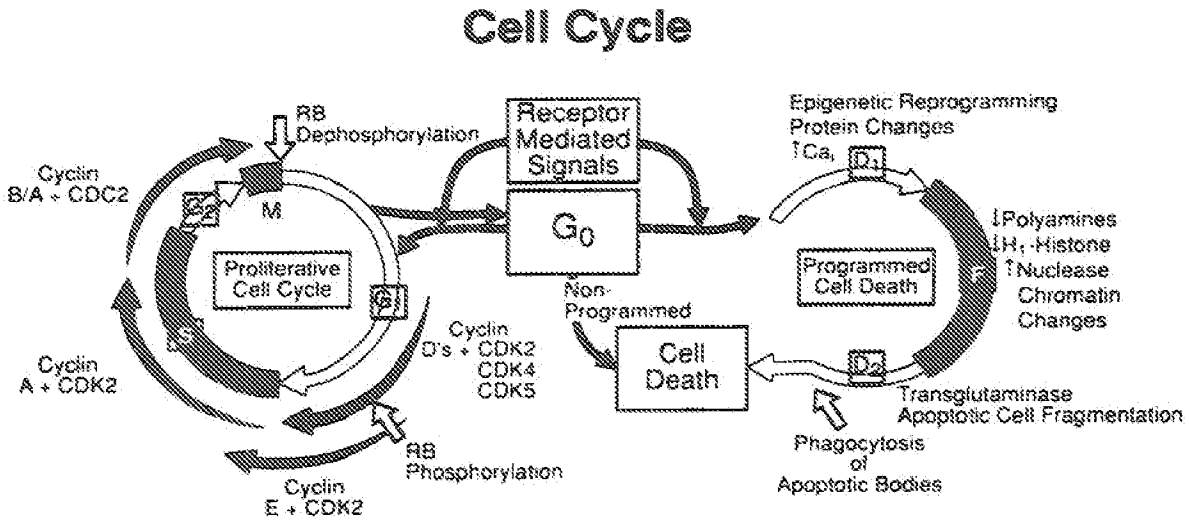

Fig. 1. Revised cell cycle denoting the options of a G_0 prostatic glandular cell. D1 denotes the period during which new gene and protein expression required for induction of the DNA fragmentation period (denoted F phase) occurs as part of the programmed cell death pathway. D2 denotes the period during which the cell itself fragments into apoptotic bodies as part of its programmed death.

uniquely determined as part of the differentiated phenotype of the particular cell. Thus the same growth factor (e.g., TGF-β_1) can have either agonistic or antagonistic effects within the cell cycle for different cell types. Therefore the specific details of the regulatory pathway for the cell cycle vary between different cell types.

PROGRAMMED CELL DEATH OF NORMAL PROSTATIC GLANDULAR CELLS FOLLOWING ANDROGEN ABLATION

In the normal adult prostate, the epithelial cells are continuously turning over with time [9,10]. In this self-renewing condition, the rate of prostatic cell death is balanced by an equal rate of prostatic cell proliferation such that neither involution or overgrowth of the gland normally occurs [9,10]. If an adult male is castrated, the serum testosterone level rapidly decreases to below a critical value [6,11]. As a result, the prostate rapidly involutes due to a major loss in the glandular epithelial cells, but not the basal epithelial or stromal cells of the prostate [12]. Only the glandular epithelial cells are androgen dependent and undergo programmed cell death following castration [12]. The chronic requirements for androgen by the glandular epithelia is due to the fact that androgens can act as agonists and antagonists by simultaneously stimulating the rate of cell proliferation while inhibiting the rate of cell death [6,9].

In the ventral prostate of an intact adult rat, glandular cells contain androgen receptor [13] and constitute approximately 80% of the total cells [12]. Approximately 70% of these glandular cells die by 7 days postcastration [12]. Using the ventral prostate of the rat as a model system, the temporal sequence of events involved in the programmed cell death pathway induced by androgen ablation has begun to be defined. In the androgen-maintained ventral prostate of an intact adult male rat, the rate of cell death is very low, approximately 1–2% per day; this low rate is balanced by an equally low rate of cell proliferation, also 1–2% per day [9,10]. If animals are castrated, the serum testosterone level drops to less than 10% of the intact control value within 2 hr [6]. By 6 hr postcastration, the serum testosterone level is only 1.2% of intact control [6]. By 12–24 hr following castration, the prostatic dihydrotestosterone (DHT) levels (i.e., the active intracellular androgen in prostatic cells) are only 5% of intact control values. This lowering of prostatic dihydrotestosterone (DHT) leads to changes in nuclear androgen receptor function (i.e., by 12 hr after castration, androgen receptors are no longer retained in biochemically isolated ventral prostatic nuclei) [6]. While the lowering of prostatic DHT and resultant androgen receptor changes are maximal by 24 hr postcastration, the programmed death of the prostatic glandular cells occurs continuously during the first 2 weeks postcastration.

These observations demonstrate that the reduction of occupancy of the androgen receptor by DHT is not

sufficient alone to activate programmed cell death of the glandular cells. Likewise, the temporally asynchronous nature of the death demonstrates that activation of programmed death of glandular cells is initiated when some other cellular survival factor besides DHT, whose level is regulated by DHT, decreases to a critical level. An excellent candidate for such a DHT-dependent survival factor is the androm-edin peptide factor, keratinocyte growth factor, normally produced and secreted by prostatic stromal cells under the stimulation of androgen [14]. Once the level of such peptide survival factors decreases to below a critical level within a particular glandular cell, a major epigenetic reprogramming of this cell occurs, resulting in the activation phase (D1) of the programmed death pathway (Fig. 1).

During this D1-activation phase, (D1a phase) (Fig. 2), the earliest events that can be seen upon androgen withdrawal are inhibition of glandular cell proliferation [15] coupled with a generalized atrophy of these secretory cells in individual acini [16,17] (Fig. 2). Universally, tall columnar secretory cells rapidly shrink and become cuboidal in shape within 24 hr of androgen deprivation. Concurrent with these global morphological changes is the initial downregulation of a series of proteins (described later). At this stage the process is completely reversible simply by replacement of exogenous androgen [18,19]. After this point, individual cells stochastically enter the D1b phase (Fig. 2) during which the activated cells morphologically round up and undergo changes in nuclear chromatin structure. During this phase, a series of proteins become upregulated and polyamine levels decrease [20]. An increase in intracellular calcium levels also occurs that appears to be derived from extracellular pools [21,22]. The mechanism for the induced change in intracellular calcium is not fully known; however, there are indications that enhanced expression of TGF-β_1 mRNA and protein [23] as well as the receptor for TGF-β_1 [23] following castration are somehow involved.

With continued androgen deprivation, prostatic glandular epithelial cells undergo a further series of changes that result in an irreversible progression through the programmed cell death pathway. During the D1b phase (Fig. 2), Ca^{2+}/Mg^{2+}-dependent endonuclease present within the nuclei of the prostatic glandular cells are enzymatically activated [21]. Levels of both histone H_1 and polyamines are decreased during this D1b phase [24,25]. Both are involved in maintaining DNA compaction [26,27]; a decrease in their respective levels allows for opening of the genomic DNA conformation particularly in the interlinking region between nucleosomes. Once this occurs, DNA fragmentation begins at sites located between nucleosomal units (i.e., F phase of Fig. 2) and cell death is no longer reversible. Recent unpublished studies using inverted pulse-gel electrophoresis have demonstrated the initial DNA fragmentation produces 300- to 50-kb DNA pieces. Once formed, these 300- to 50-kb size pieces are further degraded into nucleosomal size pieces (i.e., >1 kb). During F phase, the plasma and lysosomal membranes are still intact and mitochondria are still functional [12].

Subsequent to F phase, proteases are activated during the D2a phase, including the ICE-like proteases that hydrolyzes poly(ADP-ribose) polymerase (PARP) [28,29]. In addition, other ICE-like proteases degrade the laminins in the nuclear membrane and the nucleus itself undergoes fragmentation [28,29]. Subsequent to the nuclear fragmentation, plasma membrane blebbing, and cellular fragmentation into clusters of membrane-bound apoptotic bodies occur. This D2b phase involves an upregulation in the Ca^{2+}-dependent tissue transglutaminase activity which crosslinks various membrane proteins [30]. Once formed, these apoptotic bodies are rapidly phagocytized, during the D2c phase, by macrophages and/or neighboring epithelial cells [12,16]. This phagocytosis is induced by changes in the membrane phospholipids in the apoptotic cell and cell bodies recognized by the phagocytic cells [31]. Thus, within 7–10 days postcastration 80% of the glandular epithelial cells die and are eliminated from the rat prostate [12].

PROSTATE GENE EXPRESSION DURING PROGRAMMED CELL DEATH PATHWAY INDUCED BY ANDROGEN ABLATION

The expression of a series of genes are upregulated during the D1b phase of programmed death by prostatic glandular cells induced by androgen ablation (Table I). TRPM-2, [38] calmodulin [39], and tissue transglutaminase [30] have also previously been demonstrated to be induced in a variety of other cell types undergoing programmed cell death. At the same time, several of the genes (i.e., *c-myc*, *H-ras*) have previously been demonstrated to be involved in cell proliferation. Thus, as a comparison, the relative level of expression of these same genes was determined during the androgen-induced proliferation regrowth of the involuted prostate in animals previously castrated 1 week before beginning androgen replacement. These comparative results demonstrate that the expression of *c-myc*, *H-ras*, and tissue transglutaminase are enhanced both in prostatic cell death and in proliferation [37]. By contrast, the expression of calmodulin, TRPM-2, TGF-β_1 [37], glutathione S-transferase subunit Yb_1 [33], and α-prothymosin

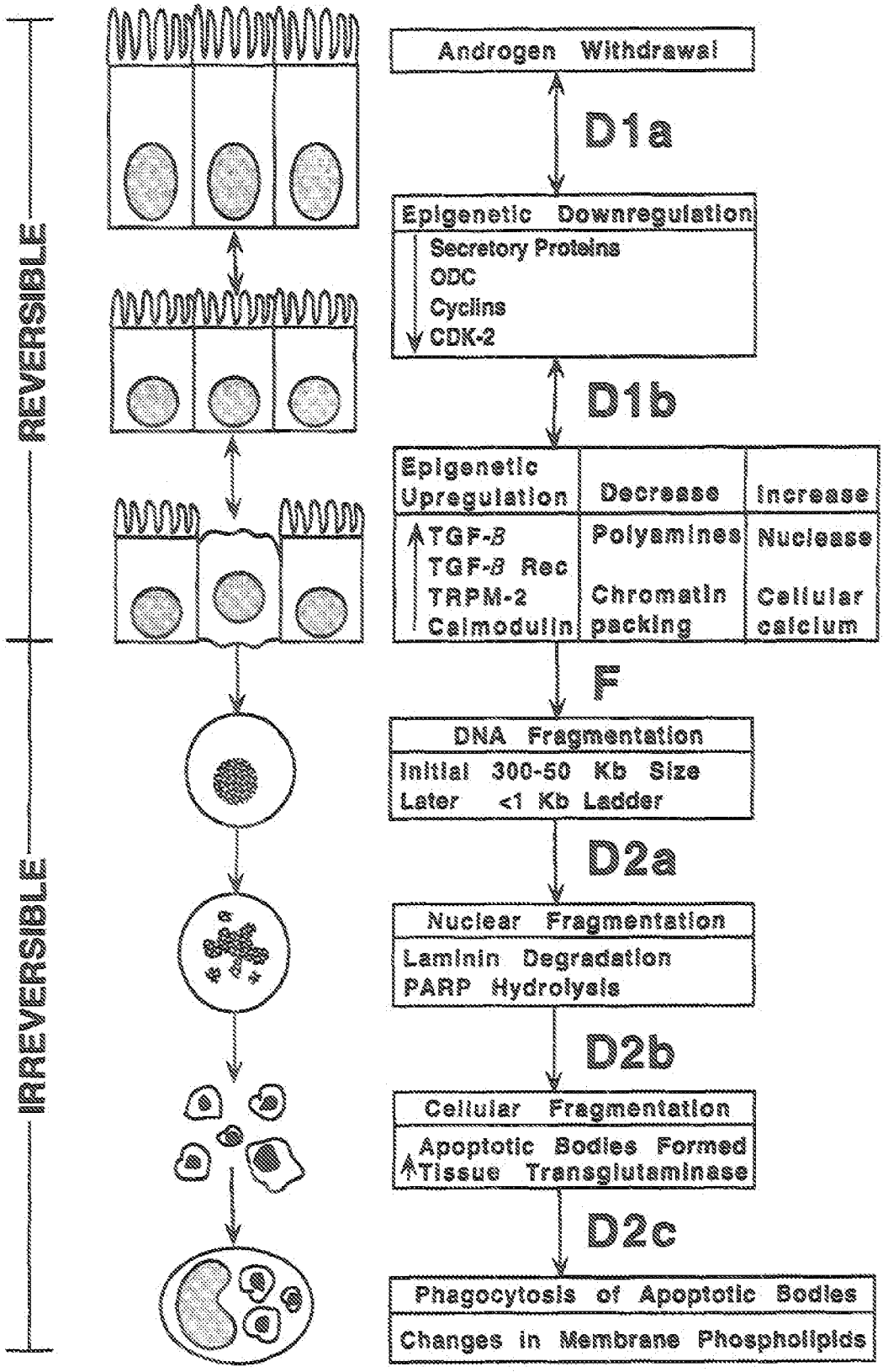

Fig. 2. Schematic diagram of the biochemical and morphological events occuring during the different phases of programmed death of normal prostatic glandular epithelium and prostatic cancer cells. (See text for specific details.)

Table I. Epigenetic Response in the Rat Ventral Prostate During Glandular Cell Proliferation/Death Induced by Androgen Manipulation[32–36]

Genes	Changes in mRNA expression during	
	Proliferation	Programmed cell death
Ornithine decarboxylase	Induced	Decreased
Thymidine kinase	Induced	Decreased
H_4-histone	Induced	Decreased
c-fos	Induced	Decreased
Glucose-reg. protein 78kDa	Induced	Decreased
Cyclin C	Induced	Decreased
Cyclin D_1	Induced	Decreased
Cyclin E	Induced	Decreased
DNA polymerase α	Induced	Decreased
C_3-prostatein	Restored	Decreased
TRPM-2	Repressed	Induced
$TGF\beta_1$	Repressed	Induced
Calmodulin	Decreased	Induced
α-prothymosin	Decreased	Induced
c-myc	Induced	Induced
H-*ras*	Induced	Induced
Tissue transglutaminase	Induced	Induced

[37] are enhanced only during prostatic cell death, and not prostatic cell proliferation (Table I).

Additional analysis demonstrated that the expression of a series of genes are decreased during the D1a phase following castration (Table I). For example, the C_3 subunit of the prostatein gene (i.e., the major secretory protein of the glandular cells), ornithine decarboxylase (ODC), histone-H_4, p53, and glucose-regulated protein 78 all decrease following castration [37]. In contrast to the decrease in the mRNA expression of these latter genes during programmed cell death in the prostate following castration, the expression of each of these genes is enhanced during the androgen-induced prostatic cell proliferation [37].

ROLE OF CELL PROLIFERATION IN PROGRAMMED CELL DEATH PROCESS INDUCED BY CASTRATION

Using the terminal transferase end-labeling technique of Gavrieli et al. [40] to histological detect prostatic glandular cells undergoing programmed death and adjusting for the half-life of detection of these dying cells, the percentage of glandular cells dying per day via programmed death in the prostate of intact and castrated rats was determined [8]. In intact (non castrated) rats, 1.2% of the glandular cells die per day via programmed death. Within the first day following castration, this percentage increases and at days 2–5 postcastration, ~17–21% of these glandular cells die per day via programmed death. These results demonstrate that both the normal constitutive and androgen ablation-induced elimination of glandular cells in the prostate is due to the programmed cell death, and not to cellular necrosis.

Using standard in vivo ^{3}H-thymidine pulse labelling, the percent of glandular cells entering the S phase during the period of enhanced prostatic cell death that occurs during the first week postcastration was determined. Within 1 day following castration, there is an 80% decrease ($P<0.05$) in the percentage of glandular cells entering S phase. By 4 days following castration, there is more than a 90% reduction in this value. Comparing the data demonstrates that greater than 98% of prostatic glandular cells die following castration without entering the proliferative cell cycle. These results confirm the previous studies of Stiens and Helpap [41] and Evans and Chandler [42], which likewise demonstrated a decrease in the percent of prostatic glandular cells in S phase following castration.

During programmed cell death activated by castration double-stranded DNA fragmentation of genomic DNA occurs and induces a futile process of DNA repair while cells remain in G_0. This futile process of G_0 DNA repair has been shown to only be associated with, but not causally required for, prostatic cell death. This was demonstrated by treating intact male rats with tridaily hydroxyurea for 1 week, which inhibits both prostate-specific DNA synthesis and unscheduled G_0 DNA repair by more than 90% for 8 hr following an intraperitoneal injection [8]. Castration of these rats resulted in similar reductions in DNA content and identical glandular morphologic changes, as compared to untreated, castrated controls. These results confirm that programmed cell death of prostatic glandular cells induced by androgen ablation does not require progression through S phase or G_0 DNA repair.

To determine whether androgen ablation-induced programmed cell death of prostatic glandular cells involves recruitment of nonproliferating cells into early portion of G_1 of a perturbed proliferative cell cycle, rat ventral prostates were assessed temporally following castration for several stereotypical molecular stigmata of entry into the proliferative cell cycle [43]. Northern blot analysis was used to assess levels of transcripts from genes characteristically activated: (1) during the transition from quiescence (G_0) into G_1 of the proliferative cell cycle (cyclin D_1, and cyclin C); (2) during the transition from G_1 to S (cyclin E, cdk2, thymidine kinase, and H_4 histone); and (3) during

progression through S (cyclin A). While levels of each of these transcripts increased as expected in prostatic glandular cells stimulated to proliferate by administration of exogenous androgen to previously castrated rats, levels of the same transcripts decreased in prostatic glandular cells induced to undergo programmed cell death following androgen withdrawal [43]. Likewise, androgen ablation-induced programmed cell death of prostatic glandular cells was not accompanied by retinoblastoma (Rb) protein phosphorylation characteristic of progression from G_1 to S. This is consistent with a decrease in the number of cells entering S cells using ^{3}H-thymidine radioautography. Nuclear run on assays demonstrated that there is no increase in the prostatic rate of transcription of the c-*myc* and c-*fos* genes following castration. Northern and Western blot analysis also demonstrated that there is no increase in the prostatic p53 mRNA or protein content per cell following androgen ablation. Likewise, following castration there is no enhanced prostatic expression of the WAF1/CIP1 gene, a gene whose expression is known to be induced by either increased p53 protein levels or entrance into G_1 [43]. These results demonstrate that prostatic glandular cells undergo programmed cell death in G_0 without recruitment into G_1 phase of a defective cell cycle and that an increase in p53 protein or its function are not involved in this death process [8,43].

To investigate further the possible role of the p53 gene in the programmed cell death pathway induced by androgen ablation, the extent of programmed death of androgen-dependent cells in the prostate and seminal vesicles following castration was compared between wild-type and p53-deficient mice. The mutant mice were established using homologous recombination to produce null mutation in both of the p53 alleles [44]. These homozygous null mutations prevent any production of p53 protein in these mice [44]. Wild-type (i.e., p53 expressing) mice and p53-deficient mice were castrated, and after 10 days the animals were killed and their seminal vesicles and prostates removed, weighted, and DNA content determined. Histological sections were also prepared from each of these tissues. These analyses demonstrated that there is an identical decrease in the wet weight and DNA content in both the seminal vesicles and prostate from wild-type and p53-deficient mice [8]. Histological analysis likewise demonstrated an identical degree of cellular regression in these tissues in the two types of mice (i.e., similar percent of terminal transferase end-labeled prostatic glandular cells in the two groups of animals). These studies demonstrate that androgen ablation-induced programmed death of androgen dependent cells does not require any involvement of p53 protein expression [8].

CELL KINETICS DURING PROGRESSION OF PROSTATE CANCER

Growth of a cancer is determined by the relationship between the rate of cell proliferation and the rate of cell death. Only when the rate of cell proliferation is greater than cell death does tumor growth continue. If the rate of cell proliferation is lower than the rate of cell death, regression of the cancer occurs. Metastatic prostate cancers, like the normal prostates from which they arise, are sensitive to androgenic stimulation of their growth. This is due to the presence of androgen-dependent prostatic cancer cells within such metastatic patients. These cells are androgen dependent, since androgen stimulates their daily rate of cell proliferation (i.e., Kp) while inhibiting their daily rate of death (i.e., Kd) [20]. In the presence of adequate androgen, continuous net growth of these dependent cells occurs because their rate of proliferation exceeds their rate of death. By contrast, following androgen ablation, androgen-dependent prostatic cancer cells stop proliferating and activate programmed cell death [20]. This activation results in the elimination of these androgen-dependent prostatic cancer cells from the patient, since under these conditions their death rate value now exceeds their rate of proliferation. Because of this elimination, 80–90% of all men with metastatic prostatic cancer treated with androgen ablation therapy have an initial positive response. Eventually, all these patients relapse to a state unresponsive to further anti-androgen therapy, no matter how completely given [45]. This is due to the heterogeneous presence of androgen-independent prostatic cancer cells within such metastatic patients. These latter cells are androgen independent, as their rate of proliferation exceeds their rate of cell death even after complete androgen blockage is performed [46].

Attempts to use non-androgen-ablative chemotherapeutic agents to adjust the kinetic parameters of these androgen-independent prostatic cancer cells, so that their rate of death exceeds their rate of proliferation have been remarkable in their lack of success [47]. The agents tested in patients failing androgen ablation have been targeted at inducing DNA damage directly or indirectly via inhibition of DNA metabolism or repair. These agents are thus critically dependent on an adequate rate of proliferation to be cytotoxic [48]. In vitro cell culture studies have demonstrated that when androgen-independent, metastatic, prostatic cancer cells are rapidly proliferating (i.e., high Kp value), these cells are highly sensitive to the induction of

programmed cell death via exposure to the same antiproliferative chemotherapeutic agent, which are of limited value when used in vivo in prostatic cancer patients [49]. The paradox between the in vitro and in vivo responsiveness to the same chemotherapeutic agents by androgen-independent prostatic cancer cells is due to major differences in the rate of proliferation occurring in the two states. Likewise, for chemotherapeutic agents to be effective, the cancer cells must have not only a critical rate of proliferation, but also a critical sensitivity to induction of cell death [50]. The sensitivity to induction of cell death is reflected in the magnitude of the rate of cell death in the untreated condition.

The daily rates of cell proliferation (i.e., Kp) and cell death (i.e., Kd) were determined for normal, premalignant, and cancerous prostatic cells within the prostate, as well as for prostatic cancer cells in lymph node, soft tissue, and bone metastases from untreated and hormonally failing patients [50]. These data demonstrate that normal prostatic glandular cells have an extremely low (i.e., <0.20% per day), but balanced, rate of cell proliferation and death producing a turnover time of 500 ± 79 day for these cells. Initial transformation of these cells into high-grade intraepithelial neoplasia (PIN), the lesion believed to be precursor for prostate cancer, results in an increased Kp value with no change in the Kd value. As these early lesions continue to grow into late-stage high-grade PIN, their Kd increases to a point equaling Kp. This results in cessation of net growth, while inducing a sixfold increase in the turnover time (i.e, 56 ± 12 days) of these cells, increasing their risk of further genetic changes. The transition of late-stage high-grade PIN cells into growing localized prostatic cancer cells involves no further increase in Kp but is due to a decrease in Kd, resulting in a mean doubling time of 479 ± 56 days. Metastatic prostatic cancer cells within lymph nodes of untreated patients have a 100% increase in their Kp and 40% decrease in their Kd values, as compared to localized prostatic cancer cells producing a mean doubling time of 33 ± 4 days. Metastatic prostatic cancer cells in the bony untreated patients have a 36% increase in Kp and a 50% decrease in Kd, resulting a mean doubling time of 54 ± 5 days. In hormonally failing patients, there is no further change in Kp. An increase in the Kd for androgen-independent prostatic cancer cells is observed within soft tissue or bone metastases with resulting mean doubling times of 126 ± 21 and 94 ± 15 days, respectively, in these metastic sites. These data demonstrate that the proliferation rate for androgen-independent metastatic prostatic cancer cells is very low (i.e., <3.0%/day), [10] explaining why antiproliferative chemotherapy has been of such limited value against metastatic prostatic cells. Based on this realization, what is needed is some type of cytotoxic therapy that induces the death of androgen-independent prostate cancer cells without requiring the cells to proliferate.

THERAPEUTIC IMPLICATION OF PROGRAMMED CELL DEATH FOR PROSTATIC CANCER

Using the human PC-82 prostatic xenograft system as a model, Kyprianou et al. [51] demonstrated that androgen ablation activates the pathway of programmed cell death, not only in normal androgen-dependent prostatic cells, but also in androgen-dependent human prostatic cancer cells. Using bromodeoxyuridine incorporation into DNA to label human PC-82 prostatic cancer cells undergoing entrance into the S phase of the proliferative cell cycle, within 1 day following castration the number of PC-82 prostatic cancer cells entering the S phase declined from 8–10% to one-third this initial values (i.e., to a value 2–3%) and that after 2 days, the proliferative activity declined to below 1%(unpublished data). Combining these latter two studies demonstrated that the programmed death of androgen-dependent human prostatic cancer cells induced by androgen ablation does not require these cells to go through a defective cell proliferation cycle but that these cells die without leaving G_0.

Additional studies have demonstrated that androgen ablation does not induce this programmed death process in androgen-independent prostatic cancer cells due to a defect in the initiation step [51]. Even with this defect, androgen-independent prostatic cancer cells retain the basic cellular machinery to undergo this programmed cell death pathway. This was demonstrated by using a variety of chemotherapeutic agents that arrest proliferating androgen-independent prostatic cancer cells in various phases of the proliferative cell cycle (e.g., G_1, S, or) and that subsequently induce their programmed (i.e., apoptotic) death [51]. One explanation for the inability of androgen ablation to induce programmed death of androgen-independent prostatic cancer cells is that such ablation does not induce a sustained elevation in the intracellular free Ca^{2+} (Ca_i) levels in these cells.

The involvement of an increase in intracellular free calcium in castration-induced prostatic cell death was indirectly inferred from studies in which rats were castrated and their ventral prostates were immediately implanted with either a placebo or a time released pellet containing the calcium channel blocker nifedipine [22,52,53]. Nifedipine is an L type (i.e., slow) calcium channel blocker that inhibits the volt-

age gated influx of calcium from extracellular pools [54]. The temporal pattern of castration-induced prostatic involution is significantly slowed in nifedipine-treated compared to placebo-treated castrated group. This nifedipine-induced delay in prostatic cell death is evident at days 3–7 postcastration [22,52]. In the study by Martikainen and Isaacs [22], the castrated, nifedipine-treated group histologically showed involution of cuboidal glandular epithelial cells like the castrated controls; however, the overall incidence of apoptotic bodies was distinctively reduced in the nifedipine-treated group. Likewise, the degree of DNA fragmentation was also significantly decreased as compared to the castrated-placebo group, and this inhibition correlates well with the degree of inhibition obtained in prostatic weight and DNA loss by nifedipine [22]. It has also been shown that nifedipine treatment of castrated animals, while not preventing induction of certain genes such as TRPM-2, did inhibit induction of c-*fos* [52] as well as a series of castration-inducible cDNAs that may be involved in an apoptosis response gene program in prostate cancer cells [53]. These studies demonstrated that treatments that inhibit a rise in prostatic intracellular free Ca^{2+} concentration derived from extracellular pools of Ca^{2+} decrease the rate of programmed cell death induced by androgen ablation. As a corollary to these inhibition studies, it has also been demonstrated that agents that increase intracellular Ca^{2+} (i.e., calcium ionophores) can activate prostatic programmed cell death, even in the presence of androgen [22].

Therefore, androgen-independent prostatic cancer cells may not undergo programmed cell death secondary to androgen ablation because such withdrawal does not induce an elevation of intracellular Ca^{2+} in these cells. To test this possibility, androgen-independent, highly metastatic Dunning R-3327 AT-3 rat prostatic cancer cells were chronically exposed in vitro to varying concentrations of the calcium ionophore ionomycin to sustain various levels of elevation in the their Ca_i [55]. These studies demonstrated that an elevation of Ca_i from a starting value of 35 nM to a value as small as only threefold above baseline (i.e., 100 nM) while not inducing immediate toxicity (i.e., death within ≤ hr) can induce the death of the cells if sustained for >12 hr. Temporal analysis demonstrated that elevation in Ca_i results in these cells arresting in G_0 within 6–12 hr following ionomycin exposure. Over the next 24 hr, these cells begin to fragment their genomic DNA initially into 300- to 50-kb size pieces, which are further degraded into nucleosome-size pieces; during the next 24–48 hr, these cells undergo cellular fragmentation in apoptotic bodies [55]. Associated with this programmed cell death is an epigenetic reprogramming of the cell in which the expression of a series of genes (to be presented later) is specifically modified. These results demonstrate that even nonproliferating androgen-independent prostatic cancer cells can be induced to undergo programmed cell death if a modest elevation in the intracellular free Ca^{2+} is sustained for a sufficient time. Combining these latter ionomycin data with the chemotherapy data demonstrates that programmed death of androgen independent prostatic cancer cells can be induced in any phase of the cell cycle and does not necessarily require progression through the proliferation cell cycle (i.e., proliferation independent).

Bcl-2 EXPRESSION AND PROGRESSION OF PROSTATE CANCER TO AN ANDROGEN-INDEPENDENT STATE

The mechanism whereby prostate cells survive androgen ablation and become androgen independent is not entirely clear. Androgen-independent prostate cancer cells maintain the ability to undergo programmed cell death; thus, it appears that androgen withdrawal fails to initiate the programmed cell death pathway in these cells [51]. Possible mechanisms for this failure could involve increased expression of genes associated with enhanced cellular survival such as bcl-2 or mutations in genes such as TP53 that may be involved in triggering programmed cell death in response to injury or DNA damage.

The bcl-2 gene is located on chromosome 18q21 and encodes a membrane-bound 26-kD protein that resides primarily in the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum [56–58]. bcl-2 has been demonstrated to be an oncogene in that its induced overexpression can lead to malignant transformation [59,60]. bcl-2 is unique among the oncogenes, however, in that its expression does not enhance the rate of cell proliferation but instead decreases the rate of programmed cell death [61,62]. The mechanism for this inhibition of programmed cell death is believed to be through the ability of bcl-2 to heterodimerize with a series of "death" proteins. One such death protein is termed bcl-2-associated x-protein, or BAX [63,64]. When BAX is allowed to homodimerize within the cell, the rate of programmed cell death is enhanced [63,64]. The antiapoptotic effect of bcl-2 may thus be via inhibition of such homodimerization through heterodimerization with BAX [63,64]. bcl-2 protein expression decreases the rate of programmed cell death by malignant hematopoietic, lymphopoietic and neuroblastoma cells induced by a wide variety of chemotherapeutic drugs [65–68]. Enhanced expression of the bcl-2 protein likewise can inhibit programmed cell death of epithe-

lium-derived cells. For example, Levin et al. [69] demonstrated that overexpression of the bcl-2 protein induced by DNA transfection of an exogenous bcl-2 gene in the Dunning AT-3 rat prostatic cancer cells prevents this programmed death initiated by lytic infection with the RNA Sindbis virus.

Within the epithelial compartment of the normal prostate, bcl-2 is expressed by the basal epithelial cells, neuroendocrine cells, and the intraacinar lymphocytes, but not by the glandular epithelial cells [70–72]. These bcl-2-negative glandular cells are the major androgen-dependent cell type present within the gland, and these cells are also the cells of origin for most human prostate adenocarcinoma [73]. McDonnell et al. [70] initially reported that there is a significantly higher frequency of bcl-2 expression in metastatic deposits of androgen-independent human prostatic cancer cells. Similarly, Colombel et al. [71] confirmed the enhanced frequency of bcl-2 expression in hormone-refractive prostate cancer. Shabaik et al. [72] have demonstrated that the malignant progression of normal prostate glandular cells to either high-grade PIN or localized stage B prostate cancer is rarely (0% and <7%, respectively) associated with bcl-2 protein expression. Finally, in a more extensive study, Furuya et al. [74] demonstrated that 17% of hormonally untreated patients with pathologically disseminated stage D1 disease were bcl-2 positive. In stage D2 disease, 53% of hormonally untreated and 42% of hormonal failure patients had bone metastases that stained positive for bcl-2 with no statistical difference seen between the two groups [74]. In this study there was no correlation between histological Gleason grade and bcl-2 expression [74].

In this same study, Furuya et al. [74] determined bcl-2 expression in a series of eight Dunning R-3327 rat prostatic cancer sublines. The cancer cells in the slowest growing, androgen-responsive, nonmetastatic H subline did not have detectable bcl-2 expression. If male animals bearing these tumors are castrated, the H tumor stops net growth for 1–2 months and then relapse occurs with the continued growth of a subset of pre-existing androgen-independent cancer cells, termed the HIS subline. While androgen independent, the HIS subline also does not have detectable bcl-2 expression. A second subline, the G subline, is composed of androgen-responsive, but not -dependent, cells and, like the H subline, does not express bcl-2. However, when animals bearing the G tumor are castrated the growth rate slows but does not stop and these slow-growing tumors now stain positive for bcl-2. Upregulation of bcl-2 following castration may explain why these cells are only androgen responsive and not truly androgen dependent. Finally, 4 out of 6 (67%) of the androgen-independent cell lines tested bcl-2 positive, indicating a statistically significant association of bcl-2 expression with progression to androgen independence [74].

These data demonstrate that, in both human and rat prostate cancer, progression of localized cancer to the metastatic, androgen-independent phenotype is associated with increased frequency of bcl-2 expression [70–72,74]. However, bcl-2 expression is not an absolute requirement for progression as 83% of lymph node metastases, and 47% of bone metastases in hormonally untreated patients were negative for bcl-2, while 58% of bone metastes were negative for bcl-2 in patients failing hormonal therapy [74].

Why bcl-2 is only expressed in some, but not all, androgen-independent metastatic tumors is unclear, but several explanations are conceivable. First, there could be multiple pathways for progression to an androgen-independent metastatic phenotype by prostatic cancer cells, only one of which is effected by bcl-2 expression. Alternatively, bcl-2 expression may not have a direct ability to induce the specific development of an androgen-independent, metastatic phenotype, but instead have an indirect ability to increase survival (clonal expansion) of random genetic variants developing stoicastically due to the genetic instability of prostatic cancer cells [75,76]. Since some of these newly developing clones could have the genetic changes required for the androgen-independent metastatic phenotype, the increased survival of such novel clones stoicastically via bcl-2 expression would indirectly increase the progression to a more malignant phenotype.

Consistent with this indirect mechanism, Furuya et al. [74] demonstrated that when bcl-2 nonexpressing androgen independent, highly metastatic AT-3 rat prostatic cancer cells were genetically engineered to overexpress bcl-2, these cells acquired an increased resistance to a variety of noxious insults. The mechanism for how bcl-2 expression induces such a multidrug cross-resistant state is unknown. In this system, bcl-2 expression did not change the kinetics or extent of early (within the first 24 hr) mRNA induced by the various agents. Nor did such bcl-2 expression inhibit the early (i.e., within 30 min) elevation in Ca_i induced by ionomycin or thapsigargin (TG). bcl-2 protein expression did inhibit, however, both the kinetics and extent of DNA and cellular fragmentation into apoptotic bodies induced after 24 hr of exposure to all the agents tested. These results demonstrated that bcl-2 protein effects a late step in the biochemical cascade of programmed cell death commonly induced by all the viral and chemical agents tested. Since the initial effects of these different agents (Sindbis virus, 5-FrdU, 4HC, ionomycin, TG) are very different, this suggests that while the biochemical path-

way for initiating programmed cell death can be variable, the biochemical pathway for completing this process eventually converges on some common stereotypic step(s), at least one of which is inhibited by bcl-2 expression [77].

Finally, although bcl-2 expression appears to be associated with progression of both human and rodent prostatic cancer cells, an explanation for how this occurs is unknown. One mechanism for such bcl-2 expression during the progression of prostatic cancer could be through defects in the p53 tumor suppressor pathway. Miyashita et al. [78] have presented evidence that p53 expression decreases the expression of bcl-2 and increases the expression of Bax. Such an explanation is consistent with the demonstration by Navone et al. [79] that p53 mutations, while infrequent in early-stage human prostatic cancer (<10%), are associated with the progression of prostatic cancer from a localized to a bone metastatic androgen-independent phenotype. In this regard, it may be more than simple coincidence that 50% of such prostatic cancer bone metastases have both p53 mutation [79] and bcl-2 protein expression [74]. Studies to test directly whether p53 mutations and bcl-2 protein expression co-localized within the same human prostatic cancer cells within bone metastases are being performed by dual immunocytochemical staining. Regardless of these results, it is clear that the clinical significance of detectable expression of bcl-2 by prostate cancer cells is that it is a predictor of aggressive clinical behavior. Since bcl-2 expression does not correlate with Gleason score, immunocytochemical staining for bcl-2 expression may well be an independent, and thus useful, adjuvant to histological grading to predict the biological behavior of prostatic cancers.

PROLIFERATION-INDEPENDENT THERAPEUTIC APPROACHES FOR ANDROGEN-INDEPENDENT PROSTATIC CANCER CELLS

There are at least three cell proliferation-independent methods to increasing the death rates of androgen-independent prostatic cancer cells. The first approach is to stimulate the host immune system to evoke or enhance a cytotoxic antitumor response, since immune killing of target cell death not required the target cells to proliferate. The second approach is to block the host development of tumor blood supply. Both the growth and metastatic ability of cancers are critically dependent on the ability of the cancer cells to induce the development of new blood vessels (i.e., termed angiogenesis) [80]. If successful, such an antiangiogenic approach would limit the growth of androgen-independent prostatic cancer via hypoxia induced tumor cell death. Indeed, linomide is an orally active agent that in preclinical animal models inhibits both the development of tumor blood vessels and thus tumor blood flow [81]. Because of its antiangiogenic ability, linomide treatment inhibits both the growth of primary prostate cancers and the establishment and growth of metastatic lesions [81–83]. Using a series of rat prostate cancer sublines that differ widely in their rate of growth, androgen sensitivity, metastatic ability, and degree of morphological differentiation, the therapeutic effects of linomide have been demonstrated to be independent of the growth rate of these cancers [82].

The third approach is to use an agent that activates the programmed cell death pathway within androgen-independent prostatic cancer cells. It has been demonstrated that elevation of intracellular Ca^{2+} with ionophores can induce programmed cell death in androgen-independent cells. A second agent that has been shown to increase intracellular Ca^{2+} to induce programmed cell death in prostate cancer cell lines and may be useful in therapy is TG [84,85], a sesquiterpene γ-lactone isolated from the root of the umbelliferous plant, *Thapsia garganica* [86,87]. Studies have demonstrated that the Ca^{2+} dependence for TG effects is attributable to the fact that this highly lipophilic agent enters cells and interacts with the Ca^{2+}-ATPase present in the endoplasmic reticulum (ER) and inhibits its enzymatic activity with an IC_{50} value of 30 nM [88]. Such inhibition is not only efficient, but also highly specific, since neither the plasma membrane nor red blood cell Ca^{2+} ATPase is inhibited by TG, even at μM concentrations [88]. Recently, it has been demonstrated that sequestered Ca^{2+} in the ER is constantly "leaking" out into the cytoplasm of the cell and that the ER-Ca^{2+} ATpase is constantly pumping this free Ca^{2+} back into the sequestered stores of the ER [88]. Thus, when the cell-permeable TG inhibits the ER-Ca^{+} ATpase pump, the leaking Ca^{2+} from the ER is no longer pumped back into a sequestered form, resulting in the three- to fourfold elevation of the Ca_i. Such a primary elevation of Ca_i leads to a depletion of the ER Ca^{2+} pool and, in many cell types, this results in a signal being generated that induces a change in the permeability of the plasma membrane to extracellular divalent cations, particular Ca^{2+}, leading to an influx due to the high free Ca^{2+} concentration extracellularly (i.e., 1–3 mM) [88]. This produces a secondary elevation in the Ca_i that is sustainable (i.e, min-hours) if the TG inhibition is maintained [88]. Based on this background, the ability of TG to sustain an elevation in the Ca_i and to activate programmed cell death in androgen-independent prostate cancer cells was tested.

Initially, in vitro testing was performed on a series of androgen-independent prostatic cancer cell lines of rat (i.e., AT-3 cells) and human (i.e., TSU-pr, DU-145, and PC-3) origin. TG was shown in microsomal preparations to produce ≥95% inhibition of the ER Ca^{2+} ATPase activity of each of these cell lines [85]. Based on these results, each of these four cell lines was chronically exposed to 500 nM TG. Using Fura-2 fluorescence ratio measurements, this TG treatment resulted in a two- to threefold elevation in the Ca_i levels from baseline values within 1–2 min of initial exposure, and this elevation of Ca_i was sustainable for >24 hr [85].

Chronic exposure of each of the four distinct androgen-independent prostatic cancer cell lines to 500 nM TG was found to arrest these cells in the G_0/G_1 phase of the cell cycle. This G_0/G_1 arrest was complete by 24 hr of continuous 500 nM TG exposure. It was further demonstrated that after a 24-hr lag period, the cells begin to fragment their DNA (i.e., to sizes ≈300 kb) and that by 96 hr of treatment, ≥95% of the cells have fragmented their DNA, regardless of cell line tested [85]. The temporal pattern of DNA fragmentation was tightly correlated with the loss of clonogenic ability by the cells for each of the four cell lines (i.e., 72 hr of TG treatment required for 50% of the cells to fragment their DNA and 50% loss of their clonogenic ability) [85]. Time-lapse videomicroscopy studies demonstrated that morphological changes begin to occur within 3–6 hr of initial TG exposure. By 24 hr of TG treatment, cells are smaller in size and rounded in morphology. At 72–120 hr TG treatment, the cells undergo a period of plasma membrane hyperactivity characterized by the production of plasma membrane blebbing [85]. These surface blebs are highly dynamic, coming and going on the surface and giving appearance of the membrane boiling previously reported for ionomycin-induced programmed cell death of AT-3 prostatic cancer cells [22]. These combined results demonstrate that the initiation of DNA fragmentation is occurring in viable nonproliferating (i.e., G_0/G_1) cells from each of the four distinct androgen-independent prostatic cancer cell lines tested, 24–48 hr before these cell lyse, and that this DNA fragmentation is not the result of a loss of metabolic viability (i.e., loss of mitochondrial or plasma membrane function). By contrast, the data are consistent with the initiation of DNA fragmentation as the irreversible commitment step in the TG-induced programmed death of nonproliferating androgen-independent rodent or human prostatic cancer cells.

Analysis of mRNA expression of the series of genes previously demonstrated to be enhanced during the programmed cell death of normal prostatic cells induced by androgen ablation demonstrated that TG treatment of androgen independent prostatic cancer cells likewise leads to an epigenetic reprogramming of the cells. AT-3 rat prostatic cancer cells were treated at 0–36 h with either 500 nM TG, 10 μM ionomycin, or 100 μM 5-fluordeoxyuri-dine (5-FrdU). Previously, we have demonstrated that prostatic cancer cells must progress through the proliferative cell cycle in order for 5-FrdU to induce their programmed cell death [51]. By contrast, TG and ionomycin induce the proliferation-independent programmed death of G_0 cells. These results demonstrate that within 1 hr of either TG or ionomycin treatment, expression of several of these genes is already elevated (e.g., α-prothymosin, calmodulin, ornithine decarboxylase [ODC]) and that by 6 hr additional genes expression is enhanced (e.g., glucose-regulated protein-78 (GRP), *c-myc*). Many of these enhancements are acute, with expression decreasing at 24 hr of treatment. There are major differences in gene expression during the proliferation-independent programmed death induced by TG or ionomycin and the proliferation-dependent death induced by 5-FrU (e.g., in the latter, *c-myc*, calmodulin, and prothymosine are not induced, while *H-ras* and TRPM-2 are induced) [84,85]. These results demonstrate that the programmed death induced by all these agents involve an active epigenetic reprogramming of the cell and the pathway induced by TG is essentially identical to that induced by ionomycin, but distinct from that induced by 5-FrDU.

The aforementioned studies have served to identify the endoplasmic reticulum Ca^{2+} ATPase pump as a potential new therapeutic target for activating programmed cell death in nonproliferating, androgen independent prostatic cancer cells. Therefore, TG could represent a novel approach to treatment. However, using TG as a therapeutic agent would be difficult for two reasons. First, TG is highly lipophilic and rapidly crosses the plasma membrane of cells and would be rapidly absorbed without reaching desirable levels in the target tissue. Secondly, an agent that is capable of killing cells quiescent in G_0 would be difficult to administer systematically without excessive toxicity, since most cells in human tissues are differentiated and not proliferating. However, if TG could be derivatized to an inactive prodrug form and targeted specifically for activation by prostatic cells, it could possibly be useful as a therapeutic agent, while avoiding significant systemic toxicity.

Currently, our laboratory is focusing on developing a method to specifically target prostatic cancer cells by taking advantage of a unique attribute of these cells, the production of prostate-specific antigen (PSA). PSA has been shown to be a serine protease with chymotrypins-like substrate specificity that is enzymatically active in seminal plasma [90,91]

while enzymatically inactived in the blood serum [92]. In collaboration with Dr. Hans Lilja, (Lund University, Sweden), we are attempting to develop a prodrug form of TG that consists of a peptide carrier representing a unique proteolytic cleavage site for PSA [93]. TG is being modified to a derivative by Dr. S. Brøgger Christensen (Royal Danish School of Pharmacy, Denmark) that can easily be coupled to this carrier peptide [94]. The TG derivative will thus be proteolytically released only in the vicinity of PSA-secreting prostate cancer cells, thereby specifically targeting these cells, while avoiding systemic toxicity.

CONCLUSION

Androgen-independent prostate cancer is currently not curable with standard antiproliferative chemotherapeutic agents. Whereas the normal prostate epithelium and androgen-dependent prostatic cancers undergo programmed cell death upon androgen withdrawal, androgen-independent cancers do not. However, these androgen-independent prostatic cancer cells maintain the machinery for activating the programmed cell death cascade. A new strategy for treatment, as typified by thapsigargin, involves inducing these androgen-independent cells to undergo programmed cell death without requiring cell proliferation. Further work is needed to better characterize the programmed cell death pathway with an eye toward developing other therapeutic agents that can activate this process in prostate cancer and in human cancers in general.

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